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(71) Applicants *(for all designated States except US)*: THE UNIVERSITY OF QUEENSLAND [AU/AU]; St Lucia, QLD 4072 (AU). UNIVERSITY OF CAMBRIDGE [GB/GB]; Downing Street, Cambridge CB2 3EH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KOOPMAN, Peter, Anthony [AU/AU]; 25 Annie Street, Torwood, QLD 4066 (AU). GOODFELLOW, Peter, Neville [GB/GB]; 56 Antrim Mansions, Belsize Park, London NW3 4XU (GB).

(74) Agent: KELLY, Robin, Thomas; Fisher & Kelly, Level 1/349 Coronation Drive, P.O. Box 1477, Milton, QLD 4064 (AU).

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(54) Title: SOX-9 GENE AND PROTEIN AND USE IN THE REGENERATION OF BONE OR CARTILAGE

(57) Abstract

An isolated DNA molecule encoding a Sox-9 gene which codes for the Sox-9 polypeptide. The human Sox-9 gene has been mapped to chromosome 17 in the same region as CMPD-1, the locus for Campomelic Dysplasia (CD). Sox-9 appears to have a role in mammalian skeletal development, and is used in the treatment of diseases involving bone or cartilage deficiency.

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TITLE

SOX-9Gene and Protein and use in the regeneration of bone or cartilage.

THIS INVENTION relates to the *Sox-9* (*SOX-9* in humans) gene which appears to have a role in mammalian skeletal development and which is also related to the inherited skeletal disease syndrome Campomelic Dysplasia (CD), alternatively known as campomelic dwarfism or campomelic syndrome.

FIELD OF THE INVENTION

CD is an osteochondrodysplasia affecting 0.05-2.2 per 10,000 live births. It is characterised by congenital bowing and angulation of the long bones, together with other skeletal defects. The scapulae are very small and the pelvis and the spine show changes. One pair of ribs is usually missing. Severe anomalies of the lower cervical spine are seen. The interior part of the scapula is hypoplastic. Cleft palate, micrognathia, flat face and hypertension are also features. Various defects of the ear have been noted, affecting the cochlea, malleus, incus, stapes and tympanum. Most patients die in the neonatal period of respiratory distress which has been attributed to hypoplasia of tracheobronchial cartilage (Lee *et al.*, 1972, Am. J. Dis. Child, **124**, 485-496) and small thoracic cage (Houston *et al.*, 1983, Am. J. Med. Genet., **15**, 3-28).

The human *SOX-9* gene has been mapped to chromosome 17 within a region which also contains *CMPD1*, the locus for CD.

Chromosomal localisation of *CMPD1* was based on three independent, apparently balanced, *de novo* reciprocal translocation involving chromosome 17 (Tommerup *et al.*, 1993, Nature Genet., 4, 170-174). All three translocations had breakpoints between 17q24 and q25, distal to the growth hormone locus (*GH*) but proximal to thymidine kinase (*TK-1*). This mapping excluded previous *CMPD1* candidates *HOX2* and *COL1A1*. Mutations within the *SOX-9* gene have now been found in DNA from CD patients (Foster *et al.*, Nature,

in press; Wagner *et al.*, Cell, in press) proving that the *SOX-9* gene has a role in skeletal development. Curiously, CD is often associated with sex reversal (Hovmoller *et al.*, 1977, Hereditas, **86**, 51-62). Among 33 cases with CD and an XY karyotype, 21 were phenotypic females and two were intersexes (Houston *et al.*, 1983, *supra*). This association defines an autosomal sex-reversal locus *SRA1* at or near the *CMPD1* locus.

Recurrent observations of CD in sibs and occasional consanguinity in CD-affected families have led to the belief that CD is inherited as an autosomal recessive disorder. However, a total of five independent *de novo* chromosomal rearrangements associated with CD lends some support to a dominant, usually lethal mutation (Tommerup *et al.*, 1993, *supra*). This may explain a case of CD affecting a mother and daughter, although it is possible that the milder phenotype in these patients represents a different mutation (Lynch *et al.*, 1993, J. Med. Genet., **30**, 683-686).

The murine *Sox-9* gene has been mapped to distal mouse chromosome 11. This region contains various disease loci including *Ts*, the locus for the mouse mutant *Tail-short*.

Tommerup *et al.*, 1993, above, have noted the similarities between CD and *Tail-short* (*Ts*), which also maps between *Gh* and *Tk-1* of mouse chromosome 11 (Buchberg *et al.*, 1992, Mammal. Genome, **3**, S162-181). No sex reversal has been associated with *Ts*. It is not yet clear whether the same gene is affected in both CD and *Tail-short*. The similarity between the two phenotypes raises the intriguing possibility that the human mutation would be homozygous lethal at the blastocyst stage, with heterozygosity resulting in the campomelic phenotype.

Ts is a mouse developmental mutant first described by Morgan, 1950, J. Hered., **41**, 208-215. The mutation is semi-dominant: homozygotes die at the blastocyst stage, before or shortly after implantation (Paterson, 1980, J. Expt. Zool., **211**, 247-256).

Heterozygotes are small with kinked tails and numerous other skeletal defects. The phenotype is variable, but typical abnormalities have been described (Deol, 1961, Proc. R. Soc. Lon. B., **155**, 78-95). The short, kinked tail is caused by reduced number and dysmorphology of caudal vertebrae. Vertebral fusions and dyssymphyses also affect the presacral and sacral regions. The humerus, tibia, and to a lesser extent femur and radius are affected by shortening and in some cases bending. Anomalies of the feet are common. These include triphalangy of digit I, absence of falciform, and various digital and other fusions. Additional ribs and rib fusions, and various skull abnormalities are evident.

Despite the obvious effects on the skeletal system in *Tail-short* and CD, there is some debate as to the nature of the primary defect. *Ts* is associated with anaemia and general growth retardation appearing at day 9, two days before the first signs of skeletal abnormality appear (Deol, 1961, above). CD is associated with vascular defects and aberrant musculature (Rodiguez, 1993, Am. J. Med. Genet., **46**, 185-192) and has been mimicked in avian and amphibian embryos by teratogens affecting the nervous system (Roth, 1991, Paedr. Radiol., **21**, 220-225).

SOX-9 encodes one of a family of transcription factors related to the mammalian Y-linked testis determining factor *Sry*. The cloning of the Y-linked testis determining gene (*SRY* in humans, *Sry* in mice) in 1990 (Gubbay *et al.*, 1990, Nature, **346**, 245-250; Sinclair *et al.*, 1990, Nature, **346**, 240-244) and subsequent demonstration that its expression is sufficient to cause male development in chromosomally female (XX) mice (Koopman *et al.*, 1991, Sry. Nature, **351**, 117-121) represented a breakthrough in positional cloning and developmental biology. The protein product of *Sry* contains a 79 amino acid motif that had already been detected in several other proteins, notably the high mobility group (HMG) of nuclear proteins (Jantzen *et al.*, 1990, Nature, 344, 830-836). Several known

sequence-specific DNA binding proteins contain a similar motif. Recent evidence that *SRY* can bind directly to DNA in a sequence-specific manner (Giese *et al.,* 1992, Science, **255**, 453-456) supports the contention that *Sry* acts as a transcription factor.

When a probe corresponding to the HMG box region of human *SRY* was hybridised to Southern blots of mouse DNA, a large number of bands was seen in addition to the strongly hybridising, Y-specific band representing mouse *Sry* (Gubbay *et al.,* 1990, *supra*). These additional bands are present in both XX female and XY male, DNA, suggesting that there are genes related to *Sry* by the HMG box, present on autosomes and/or the X chromosome. Indeed, screening of cDNA libraries with an HMG box probe derived from *Sry* yielded four classes of hybridising clone, none of them Y-linked. Sequencing of these clones showed that they are highly related to each other (78-98% amino acid homology in the HMG box region) as well as to *Sry* (77-82%). They are less closely related to other mammalian genes containing HMG boxes (around 50% amino acid homology in the HMG box region). These non-Y-linked homologues of *Sry* have been named *Sox* genes (Sry-type HMG box genes). Together with *Sry*, the *Sox* genes represent a distinct family of mouse genes that appear to encode transcription factors. Western blotting using an antibody to the *SRY* HMG box suggests that the number of *SOX* genes may be as high as 50.

cDNA clones corresponding to genes dubbed *Sox-1* to *-4* were isolated from an 8.5 days *post coitum* (dpc) mouse embryo library (Gubbay *et al.,* 1990, *supra*), raising speculation that they play a role in developmental decisions in the mammalian embryo. These genes were expressed throughout the CNS at first, and later become restricted to subsets of nervous tissue such as the developing eye and ear. It appears that *Sox-1* to *-3* are involved in specifying the development of the central nervous system. *Sox-4* acts as a transcriptional activator in T-lymphocytes (van de Wetering *et al.,*

1993, EMBO J., 12, 3847-3854). *Sox-5* is expressed stage-specifically in round spermatids in the adult testis, suggesting a role in spermatogenesis, and was also shown to bind DNA *in vitro* (Denny *et al.*, 1992, EMBO J., 11, 3705-3712). Denny *et al.*, 1992, Nucleic Acids Res., 20, 2887, identified two further *Sox* sequences, *Sox-6* and *Sox-7*, but corresponding cDNAs have yet to be cloned and their expression has not been characterised.

A further 10 members of the mouse *Sox* gene family have been identified . Degenerate primers were made corresponding to highly conserved regions at the ends of the HMG box of *Sry* and known *Sox* genes. Total RNA was prepared from 11.5 days *post coitum* (dpc) mouse embryos and reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the degenerate primers. The PCR products were cloned and sequenced to reveal seven novel genes which have been called *Sox-8, -9, -10, -11, -12, -13* and *-14* (Wright *et al.*, 1993, Nucleic Acids Res., 21, 744). Three more *Sox* sequences have also been isolated (*Sox-16, -17* and *-18*) from macrophage and muscle cDNA (Layfield *et al.*, unpublished data). Sequence comparison of the mouse *Sox* gene family in regard to the HMG box indicates that the *Sox* genes fall into seven distinct sub-groups; A: *Sry*; B: *Sox-1, -2, -3* and *-14*; C: *Sox-4, -11* and *-12*; D: *Sox-5, -6* and *-13*; E: *Sox-8, -9* and *-10*; F: *Sox-7, -17* and *-18*; G: *Sox-15* and *-16*. Whether this structural sub-grouping is reflected in the functions of these genes remains to be determined, but there is every indication that *Sox* genes represent a major development gene family, similar in many respects to the *Hox* and *Pax* families of developmental genes.

The conclusion that *Sox* genes play an important role in development is reinforced by the finding that multiple *Sox* genes are present in the genomes of many non-mammalian species. Six *Sry*-related sequences have been described in the lesser black-backed gull *Larus fuscus*, nine in American alligator, five in lizards, eight in

chickens, seven in *Drosophila* and three in frogs (Griffiths, 1991, Phil. Trans. Roy. Soc. Lond. B., **244**, 123-128; Denny *et al.*, 1992, Nucleic Acids Res. above, Coriat *et al.*, 1993, PCR Meth. App., **2**, 218-222). *Sox* genes are widespread within the class *mammalia*. *Sox-3* was recently cloned in marsupials (Foster and Graves, 1994, Proc. Natl. Acad. Sci. USA., **91**, 1927-1931), and 12 human *SOX* genes have been identified (Denny *et al.*, 1992, Nucleic Acids Res., above; Farr *et al.*, 1993, Mammal. Genome, **4**, 577-584; Gozé *et al.*, 1993, Nucleic Acids Res., **21**, 2943; Stevanovic *et al.*, 1993, Human Mol. Genet., **3**, 2013-2018).

Articles by Sinclair *et al.* (1990, Nature, **346**, 240-244), Koopman *et al.* (1991, Nature, **351**, 117-121) and Goodfellow & Lovell-Badge (1993, Ann. Rev. Genet., **27**, 71-92) referred to hereinafter also confirm that *SRY* is a dominant inducer of testis development in mammals. Since the discovery of *SRY*, many other genes have been identified that encode related HMG boxes.

The identification and cloning of *SRY* depended on the investigation of the genomes of patients with sex reversal syndromes, some with chromosomal rearrangements. In addition to *SRY* on the human Y chromosome, at least five autosomal and one X-lined loci have also been linked with XY female sex reversal and the failure to develop a testis (Bernstein, R. *et al.*, 1980, J. Med. Genet., **17**, 291-300; Pelletier, J. *et al.*, 1991, Nature, **353**, 431-434; Bennett, C.P. *et al.*, 1993, J. Med. Genet, **30**, 518-520; Wilkie, A.O.M. *et al.*, 1993, Am. J. Med. Genet, **46**, 597-600; Bardoni, B. *et al.*, 1994, Nat. Genet, **7**, 497-501; Luo, X. *et al.*, 1994, Cell, **77**, 481-490). Four of these loci have been defined by the study of rare chromosomal rearrangements. Duplications of the X chromosome short arm cause XY female development (Bernstein, R. *et al*, 1980, *supra*). The sex reversal in these patients results from the presence of two active copies of *DSS* (dosage sensitive sex reversal gene) which maps to a 160 kb region of Xp21 (Bardoni, B. *et al.*, 1994, *supra*). Autosomal

loci on chromosome 9p and on 10q have been implicated by chromosomal deletions in XY females (Bennett, C.P. *et al.*, 1993, *supra*; Wilkie, A.O.M. *et al.*, 1993, *supra*). It is not known if the sex reversal in these instances is due to monosomy for dosage sensitive genes or whether the deletions reveal recessive mutations. A third autosomal locus, *SRA1*, is on chromosome 17 (Tommerup, N. *et al.*, 1993, *supra*) and, in this case, the sex reversal is associated with CD. The diagnosis of CD is not entirely straightforward. The most conspicuous feature is congenital bowing and angulation of the long bones. However, this type of bowing is also seen in other skeletal dysplasias (McKusick, V.A., 1992, Mendelian Inheritance in Man., The Johns Hopkins Press, Baltimore). Other features may include a variety of skeletal deformities associated with bone and cartilage formation. Patients usually die in the first week of life from respiratory failure, however, the severity of the phenotype is variable and a few patients are mildly affected and survive into adult life. A striking feature of CD is the associated sex reversal. To date there have been at least 121 reported cases of CD. Of those that have been karyotyped, 24 are 46,XX females, 14 are 46,XY males, 34 are 46,XY females (with a gradation of genital defects) and two are cases of ambiguous genitalia with an XY karyotype (Tommerup, N. *et al.*, 1993, *supra*; Young, I.D. *et al.*, 1992, J. Med. Genet, 29, 251-252; Houston, C.S., *et al.*, 1983, *supra*). The remaining 47 non-karyotyped cases show a skewed sex ratio of 31:16 in favour of females. Some of the sex reversed cases examined histologically exhibit gonadal dysgenesis implying that the gene(s) responsible for CD also plays a part in testis formation.

The inheritance pattern of CD is not obvious. Many reviewers have concluded that autosomal recessive inheritance is the most likely (Cremin. B.J., *et al.*, 1973, Lancet, 1, 488-489), although it is difficult to distinguish this pattern from autosomal dominant inheritance with variable penetrance. Similarly, it is not clear if the

bone malformation and sex reversal are caused by mutation of a single gene or of a pair of linked genes in a contiguous gene syndrome. Five chromosomal rearrangements associated with CD and sex reversal have been reported which localise the gene(s) responsible to the long arm of human chromosome 17 (Tommerup, N. *et al.*, 1993, *supra*; Young, I.D. *et al.*, 1992, *supra;* Maraia, R. *et al.*, 1991, Clin. Genet, 39, 401-408). Recently, Tommerup *et al.*, 1993, *supra* have refined this localisation to 17q24.1-q25.1 with *GH* and *TK* as flanking markers. A high resolution map has been constructed across this 20 Mb region using a panel of whole genome radiation hybrids. The map has been used to position the translocation breakpoint from a 46,XY,t(2;17)(q35;q23-24) sex reversed campomelic dysplasia individual (Patient E) (Young, I.D. *et al.*, 1992, *supra*).

SUMMARY OF THE INVENTION

It has now been found that DNA sequences of the *Sox-9* and *SOX-9* genes have now been elucidated and thus preparation of recombinant proteins encoded by these genes can be facilitated. An isolated DNA molecule combining these sequences and/or the recombinant proteins can be utilised therapeutically in relation to regeneration of bone or cartilage as described hereinafter.

Therefore, in one aspect, the invention provides an isolated DNA molecule comprising a DNA sequence selected from a group consisting of:

(i) a sequence of nucleotides as shown in FIG. 1;

(ii) a sequence complementary to the sequence according to (i); and

(iii) a sequence having up to 21% variation from the sequences according to (i) or (ii) which sequence is capable of hybridising thereto under standard hybridisation conditions which codes for a polypeptide of the SOX-9 type.

In another aspect, the invention provides an isolated DNA molecule comprising a DNA sequence selected from a group

consisting of:

(a) a sequence of nucleotides as shown in FIG. 8a;

(b) a sequence complementary to the sequence according to (a); and

(c) a sequence having up to 18% variation from the sequences according to (a) or (b) which sequence is capable of hybridising thereto under standard hybridisation conditions and which code for a polypeptide of the SOX-9 type.

The invention also provides recombinant proteins encoded by both the *Sox-9* gene and the *SOX-9* gene as described hereinafter.

The *Sox-9* sequence (iii) discussed above and the *SOX-9* sequence (c) discussed above correspond to hybrids of the DNA sequences shown in FIGS. 1 and 8a as such hybrids may be isolated by standard hybridisation methods as described in Sambrook *et al.* (1989, *In* Molecular Cloning: A Laboratory Manual Cold Spring Harbour Laboratory Press, New York; in particular sections 9.31 to 9.59), or direct sequence comparison.

Hybrids of the above mentioned sequences may be prepared by a procedure including the steps of:

(i) designing primers which are preferably degenerate which span at least a fragment of the relevant DNA sequences referred to above; and

(ii) using such primers to amplify said at least a fragment either from an original cDNA library or cDNA reverse transcribed from either poly A^+ RNA or total RNA which RNA is derived from an appropriate source referred to herein.

The recombinant protein may be prepared by a procedure including the steps of:

(a) ligating a DNA sequence encoding a recombinant protein of the SOX-9 type or biological fragment thereof into a suitable expression vector to form an expression construct;

(b) transfecting the expression construct into a suitable host cell;

(c) expressing the recombinant protein; and

(d) isolating the recombinant protein.

The vector may be a prokaryotic or a eukaryotic expression vector.

Suitably, the vector is a prokaryotic expression vector.

Preferably, the vector is pTrcHisA.

The host cell for expression of the recombinant protein can be a prokaryote or eukaryote.

Suitably, the host cell is a prokaryote.

Preferably, the prokaryote is a bacterium.

Suitably, the bacterium is *Escherichia coli*.

Alternatively, the host cell may be a yeast or a baculovirus.

The recombinant protein may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook *et al.*, (1989, *supra*, in particular Sections 16 and 17).

In yet another aspect, the invention provides a method of regeneration of bone or cartilage by administration of a DNA molecule or protein referred to above to a subject suffering from bone or cartilage deficiency.

Preferably the DNA molecule or protein may be injected directly into joint tissue such as knees, knuckles, elbows or ligaments. Therefore, the compounds of the invention may be utilised as a therapeutic agent in regard to treatment of cartilage or bone damage caused by disease or aging or by physical stress such as occurs through injury or repetitive strain, e.g. "tennis elbow" and similar complaints. The therapeutic agent of the invention may also be utilised as part of a suitable drug delivery system to a particular tissue that may be targeted.

Other therapeutic applications for the compounds of the invention may include the following:-

1. *Use in cartilage and/or bone renewal, regeneration or repair* so as to ameliorate conditions of cartilage and/or bone breakage, degeneration, depletion or damage such as might be caused by aging, genetic or infectious disease, wear and tear, physical stress (for example, in athletes or manual labourers), accident or any other cause, in humans, livestock, domestic animals or any other animal species;
2. *Stimulation of skeletal development* in livestock, domestic animals or any other animal species in order to achieve increased growth for commercial or any other purpose;
3. *Treatment of neoplasia or hyperplasia of bone or cartilage*, in humans, livestock, domestic animals or any other animal species;
4. *Suppression of growth of skeletal components* in livestock, domestic animals or any other animal species in order to achieve decreased growth for commercial or any other purposes; and
5. *Alteration of the quality or quantity of cartilage and/or bone* for any other purpose in any animal species including humans.

In a broader sense, the potential uses for the *Sox-9* or *SOX-9* gene or its protein product fall into two broad categories, *viz*. (1) the promotion of bone and/or cartilage differentiation and/or growth, and (2) the suppression of bone and/or cartilage differentiation and/or growth. As such the gene or its protein product (or any part or combination of parts of either), can be described as a therapeutic agent. Thus, the therapeutic agent may be *Sox-9* or *SOX-*

9 DNA or DNA fragments alone or in combination with any other molecule, *Sox-9* or *SOX-9* protein or protein fragments alone or in combination with any other molecule, antibodies to *Sox-9* or *SOX-9* alone or in combination with any other molecule, sense or anti-sense oligonucleotides corresponding to the sequence of *Sox-9* or *SOX-9* (alone or in combination with any other molecule). The method of administration of the therapeutic agent will differ depending on the intended use and on the species being treated (see Mulligan, 1993, Science, **260**, 926-932; Morgan *et al.*, 1993, Ann. Rev. Biochem., **62**, 191-217). Such methods may include:-

(i) Local application of the therapeutic agent by injection (Wolff *et al.*, 1990, Science, **247**, 1465-1468), surgical implantation, instillation or any other means. This method may be useful where effects are to be restricted to specific bones, cartilages or regions of bone or cartilage. This method may also be used in combination with local application by injection, surgical implantation, instillation or any other means, of cells responsive to the therapeutic agent so as to increase the effectiveness of that treatment. This method may also be used in combination with local application by injection, surgical implantation, instillation or any other means, of another factor or factors required for the activity of the therapeutic agent.

(ii) General systematic delivery by injection of DNA, oligonucleotides (Calabretta *et al.*, 1993, Cancer Treat. Rev., 19, 169-179), RNA or protein, alone or in combination with liposomes (Zhu *et al.*, 1993, Science, **261**, 209-212), viral capsids or nanoparticles (Bertling *et al.*, 1991, Biotech. Appl. Biochem., 13, 390-405) or any other mediator of

delivery. This method may be advantageous for all intended uses (1-5 above) whether or not the effect is intended to be targeted to specific tissues or parts of the body, and regardless of whether the intended result is the stimulation or inhibition or suppression of *Sox-9* or *SOX-9* gene or protein activity. Where specific targeting is required, this might be achieved by linking the agent to a targeting molecule (the so-called "magic bullet" approach employing for example, an antibody), or by local application by injection, surgical implantation or any other means, of another factor or factors required for the activity of the therapeutic agent, or of cells responsive to the therapeutic agent.

(iii) Injection or implantation or delivery by any means, of cells that have been modified *ex vivo* by transfection (for example, in the presence of calcium phosphate: Chen *et al.*, 1987, Mol. Cell Biochem., 7, 2745-2752, or of cationic lipids and polyamines: Rose *et al.*, 1991, BioTech., 10, 520-525), infection, injection, electroporation (Shigekawa *et al.*, 1988, BioTech., 6, 742-751) or any other way so as to increase the expression or activity of *Sox-9* or *SOX-9* (gene or protein) in those cells. The modification may be mediated by plasmid, bacteriophage, cosmid, viral (such as adenoviral or retroviral; Mulligan, 1993, Science, 260, 926-932; Miller, 1992, Nature, 357, 455-460; Salmons *et al.*, 1993, Hum. Gen Ther., 4, 129-141) or other vectors, or other agents of modification such as liposomes (Zhu *et al.*, 1993,

Science, **261**, 209-212), viral capsids or nanoparticles (Bertling *et al.*, 1991, Biotech. Appl. Biochem., **13**, 390-405), or any other mediator of modification. The use of cells as a delivery vehicle for genes or gene products has been described by Barr *et al.*, 1991, Science, **254**, 1507-1512 and by Dhawan *et al.*, 1991, Science, **254**, 1509-1512. Treated cells may be delivered in combination with any nutrient, growth factor, matrix or other agent that will promote their survival in the treated subject.

EXPERIMENTAL

Preliminary Discussion

It has now been discovered surprisingly that expression of *Sox-9* is evident at sites where the primitive mesenchyme is condensing in the early stages of cartilage formation. It is therefore proposed that the *Sox-9* gene product regulates the expression of other genes involved in chondrogenesis by acting as a transcription factor for these genes.

As will be demonstrated hereinafter, *Sox-9* is predominantly expressed in mouse embryos in mesenchymal cells as they condense to form hyaline cartilage and is switched off once chondrogenesis is complete, consistent with a determinative role in skeletal formation. Expression and chromosomal mapping of *Sox-9* suggest that it may be the gene defective in the skeletal mutant *Tail-short*.

During embryogenesis, genetic switches act to commit undifferentiated cells to their appropriate developmental pathways. Although the master regulatory genes that constitute these switches hold the key to our understanding of how embryonic development is controlled, only a few such genes have been identified in mammals. One example is the *MyoD1* gene which alone is sufficient to activate

expression of all the genes which are required to produce the muscle phenotype; introduction of *MyoD1* cDNA into undifferentiated fibroblasts converts them into myoblasts (Davis, 1987, Cell 51 987-1000). Another developmental switch gene is the Y-linked testis-determining factor *Sry* referred to above. *Sry* is responsible for directing differentiation of cells in the different gonad to form a testis; subsequent male development is due to signals produced by the mature cells of the testis. *Sry* and *MyoD1* are DNA binding proteins and *MyoD1* has been shown to bind to a site in the promoters of other muscle-specific genes and subsequently activate their transcription (Piette, 1990, Nature, 345, 353-355; Lassar, 1989, Cell 58, 823-831). *Sry* is presumed to activate transcription of genes downstream in the sex-determination pathway, although these genes have not yet been identified.

During skeletogenesis, most bones are laid down initially as a framework of hyaline cartilage. In this process, mesenchyme condenses and assumes the approximate shape of the bone, chondroblasts differentiate within this structure and extracellular matrix proteins characteristic of this type of cartilage are synthesised. These cartilage models are subsequently transformed into bone as calcium salts are deposited within them during ossification.

Characterisation of the mouse Sox-9 gene

By screening mouse embryo cDNA libraries with a *Sox-9* HMG box probe, three incomplete but overlapping clones were identified. The nucleotide and deduced amino acid sequences of a composite cDNA molecule are shown in FIG. 1. The 2249 base-pair sequence reveals an open reading frame that potentially encodes a protein of 507 amino acids from the first methionine codon. There are three other AUG codons upstream of the HMG box but only the last of these (position 26, FIG. 1) is associated with a strong consensus sequence for initiation of translation (Kozak, 1989, J. Cell Biol., 108, 229). There are multiple stop codons (not shown)

following the end of the coding sequence and a putative poly-adenylation signal AATTAAA is present 14 bases upstream of a poly-A tail. Comparison of *Sox-9* PCR product sizes from cDNA and genomic DNA templates, and sequencing of *Sox-9* genomic clones revealed two introns, one of which interrupts the HMG box domain (FIG. 1). This is the first report of introns in any member of the *Sox* gene family in the mouse, although introns have also been identified in the same positions in human and chick *Sox-9* homologues.

Sox-9 cDNA sequence 3' to the HMG box is rich in both glutamine and proline residues, a common feature amongst the activation domains of known RNA polymerase II transcription factors (van de Wetering, 1991, EMBO J., **10**, 123-132; Mermod, 1989, Cell, **58**, 741-753; Courey, 1988, Cell, **55**, 887-898; Clerc, 1988, Genes Dev., **2**, 1570-1581; Scheidereit, 1988, Nature, **336**, 551-557; Muller, 1988, Nature, **336**, 544-551; Norman, 1988, Cell, **55**, 989-1003). It has now been demonstrated that this domain of the *Sox-9* protein can function as a transcriptional activator *in vitro* using the yeast *GAL4* assay (Lillie, 1989, Nature **338** 39-44). Transcription of the CAT reporter gene was activated following co-transfection with vectors which directed expression of *GAL4/Sox-9* fusion proteins containing either the whole of the *Sox-9* open reading frame, or the putative activation domain from amino acid positions 329 to 507 (data not shown).

Expression of Sox-9 during mouse embryogenesis

Sox-9 expression was examined in whole embryos by Northern blotting of polyA$^+$ RNA. The size of the mRNA was shown to be approximately 5.5kb, indicating that there is a considerable region of 5' untranslated sequence which is not present in any of the cDNA clones. Expression of *Sox-9* mRNA was detected from 8.5 dpc through to 13.5 dpc, peaking at 12.5 dpc (FIG. 2).

Wholemount *in situ* hybridisation showed *Sox-9* expression in mesenchyme in the head and the first branchial arch,

and also in the more mature rostral somites at 9 dpc (FIG. 3a). Strongest expression at this stage occurred in the otocysts and in a scattered population of surface ectoderm cells overlying the spinal cord for a distance of several somite lengths, located near the middle of the anteroposterior axis. The significance of this latter staining is not clear, but it persists at least until 13.5 dpc, moving gradually in a caudal direction as the axis extends. At 10 dpc, intense staining was present in the facial and first branchial arch mesoderm (FIG. 3b) and expression had extended to all somites. However, in the less mature caudal somites, staining was seen in a discrete population of cells within each somite, consistent with expression in the sclerotome compartment which gives rise to the cartilage of the trunk; in the more mature rostral somites, evidence of sclerotomal migration could be seen. Intense staining persisted in the otocysts. Some signal was observed in tubular structures in the heart. Curiously, ventricular cells of the fore- and midbrain were positive, but less mature regions of the central nervous system (including hindbrain and spinal cord) were negative. This staining of the ventricular cells moved further caudally in later stages, reaching the tail by 11.5 dpc (see FIG. 3h).

At 10.5 dpc, strong staining was seen in the mesoderm surrounding the nostril invaginations (FIG. 3c). Strongly staining condensations were present in the first and second branchial arches, and also in the limb buds. The limb bud condensations acquire strong *Sox-9* expression in a very short time (no staining was observed at 10 dpc), and clearly precede the deposition of cartilage in these sites, as judged by alcian blue staining of embryos (FIG. 3d). This indicates that *Sox-9* is likely to be the cause rather than the consequence of chondrocyte differentiation. In the forelimb buds, there were in fact two distinct but overlapping condensations, the more proximal of which was presumably the humeral condensation. At this stage, *Sox-9*-positive sclerotomal cells could clearly be seen migrating from the rostral somites (FIG. 3c), but remained within the confines of the

caudal somites. Expression in the otocysts had decreased in the period 10 to 10.5 dpc, and continued to decrease subsequently. Staining was clearly visible in the notochord in the tail region posterior to the hindlimb bud; more anterior staining, if any, may have been obscured by the depth of the notochord within the embryo.

The pattern of *Sox-9* expression associated with the developing limbs became more complex in subsequent days. By 11.5 dpc, the more distal condensation had progressed to form radius, ulna and footplate condensations (FIG. 3e). In addition, a prominent girdle corresponding to the scapula was strongly positive for *Sox-9*.

The correlation between *Sox-9* expression and skeletal development was most striking at 12.5 dpc (FIG. 3f), when staining was observed in most skeletal structures visualised by alcian blue staining (FIG. 3g). *Sox-9* expression was evident in the developing vertebrae, ribs, long bones, digits and cranial cartilage. At some sites, such as where the digits were forming at 12.5 dpc, the domain of *Sox-9* expression was broader than that of the alcian blue staining, reinforcing the suggestion that *Sox-9* is expressed not only in chondrocytes but also in their condensing mesenchymal progenitor cells. At this stage the expression in the ventricular cells of the spinal cord was clearly visible as two parallel stripes when viewed dorsally (FIG. 3h).

By 13.5 dpc, *Sox-9* staining was confined to the tail-tip vertebrae, the tips of the digits, the ribs and the nasal cartilage, where chondrogenesis was still in progress, and was no longer seen where chondrogenesis was complete, for example, in the long bones of the limbs and the proximal parts of the digits (FIG. 3i). Prominent staining was also observed in the vibrissae. The staining of ventricular cells of the spinal cord was by this time only observed posterior to a point midway between the fore- and hindlimbs, apparently regressing in an anterior to posterior direction.

Experimental bone fracture induces expression of Sox-9

Wholemount *in situ* hybridisation studies using a *Sox-9* antisense probe have revealed that subsequent to experimental fracture of mouse bone in accordance with the method described in (Nakase, *et al.*, 1995, J. Bone and Min. Res., **9**, 651-659), strong expression of *Sox-9* was obtained in chondrocytes at eight days post-operation (FIG. 4) whereas there was no expression of *Sox-9* detected in control chondrocytes (data not shown). These results indicate that *Sox-9* gene expression is transiently induced by experimental bone fracture.

Linkage analysis

Using the interspecific backcross method, *Sox-9* was mapped to distal chromosome 11. Linkage analysis suggested a localisation 18.0 ± 5.4 cM from the marker D11Mit10, or 26.5 ± 6.3 cM from the marker D11Mit36 (FIG. 5). Chromosome 11 haplotype analysis of recombinants from this backcross indicates that *Sox-9* maps distal to D11Mit10. Known mouse developmental mutants that map to this region include the neurological mutants *Jackson-shaker (js), teetering (tn)* and *cerebellar outflow degeneration (cod)* (FIG. 5) (Buchberg, 1992, above). Amongst mutations in this region is *Tail-short (Ts)* referred to above. Homozygous *Ts* blastocysts are unviable but heterozygotes survive and are small with shortened, kinked tails caused by reduced number and dysmorphology of caudal vertebrae, and display a variety of skeletal abnormalities as described above. These include vertebral fusions and dyssymphyses, dysmorphology of the humerus, tibia, femur and radius, digital triphalangies and fusions, additional ribs and rib fusions and various abnormalities of the skull. The notochord, neural tube and heart are malformed. The skeletal abnormalities displayed by *Ts* mice all occur in tissues where *Sox-9* is expressed during development. In view of the mapping and expression data, *Sox-9* is a good candidate for the gene defective in *Tail-short* mice.

It has been demonstrated that *Sox-9* is involved in the

formation of the skeleton during mouse embryogenesis. It is strongly expressed at sites where skeletal components are being laid down as cartilage.

Our observations suggest that *Sox-9* expression is a cause rather than a consequence of chondrocyte differentiation. First, *Sox-9* expression precedes the deposition of cartilage in all skeletal elements. *Sox-9* expression is the earliest known marker of sclerotomal cells, the primordial cells that give rise to trunk cartilage. In the digits *Sox-9* is expressed in a broader domain than that where cartilage matrix had already been laid down, indicating that it is initially switched on in loosely packed progenitor cells and is expressed throughout the condensation process.

Secondly, expression of *Sox-9* ceases soon after deposition of cartilage; by 13.5 dpc the staining in the long limb bones and proximal ends of the digits was no longer visible, but was maintained in sites where chondrogenesis persists, such as the tail and digit tips. The short period of *Sox-9* expression suggests that *Sox-9* has a role during initiation of chondrogenesis and is no longer required once condensation is complete and cartilage-specific protein synthesis begins. The temporary expression of *Sox-9* is similar to that of the closely related testis determining gene *Sry*, and suggest that *Sox-9* may act as a genetic switch in determining the fate of the mesenchymal cells in which it is expressed.

Thirdly, it is likely that *Sox-9* functions as a transcription factor, as do the products of several other members of the *Sox* gene family. *Sox-9* contains an HMG box (a motif known to act as a site-specific DNA-binding domain) and we have demonstrated ability of its carboxyl terminus to activate transcription of a reporter gene. It therefore seems likely that *Sox-9* activates genes downstream in the chondrogenic pathway. Such genes may include regulatory molecules such as members of the bone morphogenetic protein family (reviewed by Kingsley, 1994, Trends Genet., 10, 16-21) or structural genes

such as α1 (II) collagen, which is a major component of cartilage.

The expression patterns of *Sox-9* in the developing skeleton and in other tissues, such as the notochord, central nervous system and heart, correlate with defects that occur in *Ts* embryos. In addition, mouse *Sox-9* maps to the *Ts* locus. Taken together, these data implicate *Sox-9* in the genetic defect *Tail-short (Ts)*. While our data provide a ready explanation for the skeletal defects in *Ts* mice, it is not clear how defects in *Sox-9* might explain the anaemia exhibited by *Ts* embryos (Deol, 1961, above); we were unable to detect *Sox-9* expression in the yolk sac where *Ts* mice have reduced blood islands at an early stage. The semi-dominant nature of this mutation may be due to haploinsufficiency, in which two functional copies of the gene are required to produce enough product for normal development. However, the inviability of *Ts* homozygote blastocysts implies that the gene responsible for the *Ts* defect must be aberrantly expressed at the blastocyst stage, and no expression of *Sox-9* in blastocysts was detected at 4 dpc. It is possible that *Sox-9* is expressed earlier than 4 dpc. Alternatively, the defects may be a result of overexpression or inappropriate expression directed by the mutant allele.

Expression of *Sox-9* was observed in several non-skeletal tissues both during development and in the adult. In some tissues this may be a reflection of the presence of chondrocytes. In the brain and spinal cord, *Sox-9* is clearly expressed in the rapidly dividing neurones of the ventricular zone. A common symptom of campomelic dysplasia is mental retardation, suggesting that the observed expression in the developing central nervous system, and possibly also in the adult brain, has a functional significance. We also observed expression of *Sox-9* in mouse fetal genital ridges and early gonads. As XY sex reversal often associated the campomelic dysplasia (Hovmoller, 1977, *supra*), *Sox-9*, like its Y-linked relative *Sry*, must also have a role in sex determination, at least in humans. It is not yet known whether *Sox-9* and *Sry* are expressed in the same

cell type, nor whether *Sox-9* interacts with, competes with, or acts downstream from *Sry*. Sex reversal has not been noted for *Ts* mice, and it is possible that the mutant allele involved in *Ts* does not cause the sex reversal phenotype. Gain- and loss-of-function analyses in transgenic mice will be necessary to elucidate the roles of *Sox-9* in sex determination as well as in neural and skeletal development.

HUMAN *SOX-9*

Preliminary Discussion

Adjacent to the translocation breakpoint as hereinbefore described, a human *SOX-9* has been found. Mutation analysis and sequencing of *SOX-9* in clinically confirmed campomelic patients without cytologically detectable chromosomal arrangements have identified several mutations as described hereinafter. Detailed data are presented for three patients, two with confirmed *de novo* mutations, one of which occurs in an XY female, demonstrating that mutations in this gene cause both CD and *SOX* reversal.

Construction of a high resolution map of 17q24.1-q25.1

Radiation hybrid mapping allows the integration of different types of markers into a single map (Walter. M.A. *et al.*, 1993, Trends in Genetics, 9, 352-356; Walter, M.A. *et al.*, 1994, Nature Genet., 7, 22-28). We have used PCR to screen DNA samples from a panel of 129 whole genome radiation-fusion hybrids with a total of 38 STS markers across the region from *GH* to *TK* on chromosome 17. These markers include 26 microsatellites, 2 anonymous DNA markers and 10 genes. One of the genes used as a marker, *SOX-9*, we had previously mapped to the long arm of chromosome 17 (unpublished data, see legend to FIG. 8). The same markers were then tested on the somatic cell hybrid B1, which was constructed by fusing mouse L cells with fibroblasts from E., a sex reversed CD patient. The hybrid B1 retains the human translocation chromosome 2pter-q35:17q23-qter in the absence of the reciprocal translocation chromosome and the normal chromosome 17 from the

parent cell line. Chromosome 17 markers present in B1 must be located distal to the breakpoint (i.e. between the breakpoint and the end of the long arm of chromosome 17), while markers missing from the hybrid must be located proximal to the breakpoint. From this analysis, the microsatellite marker *D17S970* was deduced to be the closest proximal marker to the breakpoint and the gene *SOX-9* was found to be the closest distal marker (FIG. 6). Assuming an approximate distance of 20 Mb between *GH* and *TK*, the radiation hybrid map can be used to estimate the distance between *D17S970* and *SOX-9* as 1-2 Mb.

Construction of a YAC contig and the precise localisation of the translocation breakpoint

The markers flanking the translocation breakpoint were used to screen the ICRF (Lehrach, H. *et al.*, 1990, *In* Genome Analysis Volume 1: Genetic and Physical Mapping (eds. Davies, K.E. & Tilghman, S.H., pp 39-81, Cold Spring Harbor Laboratory Press, Cold Spring Harbor) and CEPH YAC libraries (Cohen. D. *et al.*, 1993, J. Nature, **366**, 698-701). One the flanking STS markers (*D17S970*) and an additional marker in this region (*D17S949*), had already ben used to screen the CEPH library as part of the Genethon and Whitehead/MIT Genome Center mapping projects. The YACs identified in these screens were sized, and a YAC contig was constructed based on STS content (FIG. 7). Probes from the ends of the YACs were isolated and tested back on hybrid B1 DNA as well as the other YACs to verify the contig. The ICRF YAC D0292, which was identified by the *SOX-9* probe, yielded an end clone, D0292R, that failed to hybridise with hybrid B1 DNA. This result placed the translocation breakpoint in the region between *SOX-9* and D0292R. Analysis of D0292 by pulsed-field gel electrophoresis determined that these markers were separated by 105-120 kb (data not shown).

A cosmid contig of the region between *SOX-9* and D0292R was constructed by screening the ICRF chromosome 17

cosmid library (Lehrach, H. *et al.*, 1990, *supra*) with inter-Alu PCR products derived from one of the YACs (946 E12) which spans the region. Inter-Alu positive cosmids were tested with markers flanking the translocation breakpoint and these served as starting points for a cosmid walk. A contig was assembled using isolated cosmid ends to identify overlapping cosmids from the YAC Alu-PCR positive cosmid set (FIG. 7). The end clones were mapped back onto the hybrid B1 and one of these detected the breakpoint in Patient E and hybrid B1 on Southern blots of *Bam*HI digested DNA (data not shown). The distance from the breakpoint to the *SOX-9* open reading frame is 88 kb.

Characterisation of the SOX-9 gene

Transcripts corresponding to the human *SOX-9* gene were isolated as part of experiments aimed at identifying novel *SOX* genes by screening a testis cDNA library at high stringency with a *SOXA* HMG box probe (Stevanovic, M. *et al.*, 1993, *supra*). The isolated cDNAs were identified as *SOX-9* based on similarity to the published partial sequence containing the mouse *Sox-9* HMG box region (Wright, E.M. *et al.*, 1993, *supra*). We have assembled a composite transcript of 3934 bp using sequence obtained from cDNA clones isolated from three independent libraries (FIG. 8a). Comparison of this sequence with the corresponding genomic DNA revealed the presence of two introns (FIGS. 8a and 8b), the boundaries of which have canonical splice site junctions. *SOX-9* is the first *SOX* gene reported to contain introns; other *SOX/Sox* genes studied at the genomic level (*SRY, SOX-3* and *SOX-4* and *Sox-4*) are single exon genes (Sinclair, A.H. *et al.*, 1990, *supra*; Stevanovic, M. *et al.*, 1993, *supra*; Farr, C.J. *et al.*, 1993, *supra*; Schilham, M.W. *et al.*, 1993, Nucleic Acids Res., **21**, 2009). The 3' region of the composite cDNA sequence contains a potential polyadenylation signal located 19 bp upstream from a terminal polyadenosine tract. The cDNA sequence diverges from the genomic sequence at the poly(A) tract, indicating

that the cloned cDNA contains the 3' end of the *SOX-9* transcript. The composite cDNA contains an open reading frame (ORF) with an HMG box and three potential start codons. Using the most 5' methionine as the translation start site, a polypeptide of 509 amino acids is predicted (FIG. 8a). This methionine is located 125 bp downstream of an in-frame stop codon, strongly suggesting that the complete ORF is contained within the cloned cDNA sequences. Northern blot analysis using a *SOX-9* cDNA probe detects a transcript of approximately 4.5 kb in total cytoplasmic RNA from adult testis, adult heart and foetal brain (data not shown). The discrepancy of approximately 600 bp between the cDNA sequence length and the transcript size seen in Northern blots can be accounted for by as yet unidentified 5' non-coding sequences and polyadenylation of the transcript. The *SOX-9* protein HMG box domain at amino acids 104-182 shares 71% similarity with the *SRY* HMG box and the c-terminal third of the protein has a proline- and glutamine-rich region, similar to activation domains present in some transcription factors (Mitchell, P.J. *et al.*, 1989, Science, 245, 371-378). DNA and protein sequence database searches and subsequent sequence alignment with the *SOX-9* HMG box identified mouse *Sox-9, Sox-8* and *Sox-10* as the most related sequences at 100%, 98% and 93% predicted amino acid identity respectively. The same searches using sequences located outside the HMG box did not detect any significant matches in the databases apart from mouse *Sox-9*. The human and mouse predicted proteins share 96% identity and these differences are mostly due to conservative substitutions however there was a marginal reduction in amino acid identity between mouse SOX-9 and chicken SOX-9 (93.4% identity) and between human SOX-9 and chicken SOX-9 (93.4% identity).

At the DNA level, sequence comparison between the respective predicted coding regions of the human *SOX-9* gene and the mouse *Sox-9* gene herein described reveals that these sequences

share 91.3% identity. On the other hand, sequence comparison between these predicted coding regions and that of chicken *Sox-9* (GenBank Accession No. U12533) indicates reduced identity at the DNA level (Mouse x Chicken: 79.3%; Human x Chicken: 82.4%). These data suggest that *Sox-9* genes have higher identity within a class of vertebrates than between different classes. However, the coding regions can be subdivided respectively into several distinct sub-regions (See FIG. 9 illustrating the structure of mouse *Sox-9*). Amongst these is the HMG box (nt 608-843, FIG. 9), the highly conserved region that defines the *Sox* gene family (Goodfellow and Lovell-Badge, 1993, Annu. Rev. Genet., 27, 71-92); this region shows greater than 60% homology between all the members of the *Sox* gene family. Sequences outside this region give each *Sox* gene its individual character. Another region is a short stretch composed exclusively of proline (P), glutamine (Q) and alanine (A) reduces (nt 1322-1430, FIG. 9). Regions such as this are found in many genes, often associated with protein regions that act as transcriptional activators.

The remainder of the gene may be subdivided into three regions arbitrarily designated a, b, and c (FIG. 9). These regions are highly homologous between mouse *Sox-9* and human *SOX-9* (mammalian equivalents) (Table 1). Conversely, there is reduced homology between the respective mammalian regions and those of chicken *Sox-9* (Table 1).

The very high degree of homology between mouse and human *Sox-9* and the lack of other genes showing significant homology to *Sox-9* enables a person skilled in the art to use these mammalian *Sox-9* genes or parts thereof (preferably greater than 15 nt in length) as a means of generating other mammalian *Sox-9* homologues using high stringency library screening (Sambrook *et al.*, 1989, *supra*).

Initial localisation of *SOX-9* using a monochromosomal

somatic cell hybrid mapping panel, following by sublocalisation using chromosome 17 deletion hybrids mapped the gene to 17q23-qter (see FIG. 8 legend). This localisation was refined to 17q24 by fluorescence *in situ* hybridisation.

Mutation analysis of SOX-9

The juxtaposition of *SOX-9* and the translocation breakpoint in B1, as mapped using the radiation hybrid panel, prompted us to test for mutations in this gene in DNA samples from patients with clinically confirmed CD that do not have cytologically detectable chromosomal aberrations. Initial screening was performed using a single-strand conformation polymorphism (SSCP) assay. Primers were designed to amplify the known coding sequences and intro/exon junctions in overlapping fragments of approximately 150 bp. Fragments that gave altered SSCP patterns (unique SSCP conformers) were cloned into plasmid vectors and sequenced. Nine patient samples were investigated; these samples yielded six heterozygous mutations. We describe here three patients in detail.

Patient S.H. (46,XXfemale) (ECACC No. DD1813). This patient was delivered at full term with typical features of CD: micrognathia, hypoplastic scapulae, bilateral talipes equinovarus, hypoplastic cervical vertebrae, blowing of the long bones and eleven pairs of ribs. Cloning and sequencing of a unique *SOX-9* SSCP conformer for this individual revealed a cytidine to thymidine base transition (nucleotide 583) that introduces a stop codon at amino acid position 195 of the predicted 509 amino acid sequence (FIG. 10). Both parents of this patient were screened by SSCP for this portion of *SOX-9* and neither showed an aberrant shift (FIG. 10). In addition, DNA samples from over 100 unaffected individuals were screened by SSCP for this region of *SOX-9*. No anomalous shifts were seen in any normal individual. This is a *de novo* mutation.

Patient A.H. (46,XYfemale) (NIGMS No. GM01737). This sex reversed individual was delivered at term with a full spectrum

of CD symptoms including short bowed limbs, small scapulae and characteristic facial features (Hoefnagel, D. *et al.*, 1978, Clinical Genetics, 13, 489-499). Normal external female genitalia were present and the gonads were poorly differentiated with a substantial number of germ cells. Cloning and sequencing of the unique SSCP conformer for this patient (FIG. 10) identified a single G insertion in a series of six Gs (nucleotides 783-788) contained within codons 261-263 of *SOX-9*. The resulting frameshift introduces a premature stop codon such that a 294 amino acid protein would be translated, rather than the predicted normal 509 amino acid protein. Parental DNA of this patient could not be obtained. To investigate the possibility that this mutation occurs in unaffected individuals, SSCP was performed on this region of *SOX-9* in more than 100 individuals without CD. No shifts corresponding to the Patient A.H. unique conformer were found.

Patient G. (46,XYfemale). Following ultrasound findings of short limbs and cystic hygroma, this foetus was aborted at 17 weeks. Clinical and radiological features include micrognathia, bowing of the limbs, hypoplastic scapulae, dislocated hips and eleven pairs of ribs. Normal female genitalia were present and the ovaries histologically appear normal with oocytes. The mutation found in the unique SSCP conformer from this patient was found to be the result of a four basepair insertion following amino acid 286 (nucleotide 858) of the predicted protein sequence (FIG. 8a). This frameshift introduces a premature stop at the same position as in patient A.H. SSCP analysis of this region of *SOX-9* from both parents revealed a normal *SOX-9* shift (FIG. 10). This is a *de novo* mutation.

We have used a positional cloning approach to define a breakpoint from a patient with both CD and autosomal XY sex reversal. The open reading frame of *SOX-9*, an *SRY*-related gene, is located 88 kb distal to the breakpoint on chromosome 17. We have found mutations in single alleles of *SOX-9* in six of nine campomelic

dysplasia patients examined. The three mutations described in detail here would be expected to destroy gene function: two mutations cause frameshifts which lead to premature chain termination and loss of one third of the protein and one mutation causes a premature termination that truncates the protein at 40% of its predicted length. Control populations of greater than 100 unaffected individuals were screened for two of these mutations and none were detected. SSCP analysis of both parents of two of the patients revealed the absence of the mutation present in their offspring. The *de novo* appearance of a mutation in a sex reversed CD patient establishes that alterations in *SOX-9* can cause both campomelic dysplasia and autosomal sex reversal.

The precise relationship between the translocation breakpoint and *SOX-9* is currently unclear. The *SOX-9* transcript in adult testis, adult heart and foetal brain is approximately 4.5 kb, however, the cDNA isolated from testis, foetal brain and fibrosarcoma cDNA libraries cover 3.9 kb of the transcript, leaving approximately 600 bp of untranslated sequence unaccounted for. The genomic arrangement of *SOX-9* is such that the 5' end is oriented towards the chromosome 17 centromere and closest to the breakpoint. It is possible that one or more exons are present 5' to the known exons and that these are disrupted by the translocation. Alternatively, the translocation may disrupt expression by a more subtle mechanism, such as interfering with chromatin domains Dillon, N. *et al.*, 1994, Current Opinion in Genetics and Development, 4, 260-264). Such long-range position effects have been demonstrated for *Sry*, where deletions of Y chromosomal material outside the minimal testis determining region can disrupt *Sry* expression and cause XY female sex reversal (Capel, B. *et al.*, 1993, Nat. Genet, 5, 301-307). Other instances of genes affected by translocations located at a distance have been reported (Tommerup, N., 1993, J. Med. Genet., **30**, 713-727). It is striking that several of the CD translocation patients have

survived early childhood and the disease may be milder in these individuals (Mansour, S., 1994, MSc Thesis (Clinical Genetics), University of London).

Campomelic dysplasia has previously been described as an autosomal recessive or even X-linked disease, although a few cases are more consistent with a dominant disorder (Bianchine, J.W. *et al.*, 1971, Lacet, **1**, 1017-1018; Thurmon, T.F. *et al.*, 1973, J. Ped., **83**, 841-843; Lynch, S.A. *et al*, 1993, *supra*). Our results support the suggestion that CD is an autosomal dominant disease. We have not detected a mutation in both *SOX-9* alleles of any patient, in spite of having performed SSCP across greater than 70% of the *SOX-9* open reading frame. Although it is possible that a common null allele remains undetected, the frequency of this mutation would have to be improbably high to be found in our unrelated patients. The predicted loss of gene function in these mutants together with the absence of mutations in both alleles implies that the dominance is due to haplo-insufficiency rather than gain of function. Dosage sensitivity is often a feature of regulatory genes and has been described for several sex determination systems including the mammalian pathway (Bardoni, B. *et al.*, 1994, *supra*; Parkhurst, S.M. *et al.*, 1994, Science, **264**, 924-932)

A prediction for autosomal dominance of *SOX-9* mutations is that deletions resulting in monosomy 17q should cause CD. Such deletions are very rare, presumably due to an associated lethality and have nearly always been reported associated with a ring chromosome. Interestingly, in a single reported 17q deletion not associated with a ring chromosome, the patient exhibited a number of physical features that occur in CD, including angulation of the lower limbs (Bridge, J. *et al.*, 1985, Am. J. Med. Genet, **21**, 225-229). Cases diagnosed as CD have a wide range and severity of associated phenotypes, including "acampomelic" campomelic dysplasia and the suggestion of long bone and short bone varieties (McKusick, V.A.,

1992, *supra*). It will be of interest to determine the extent of *SOX-9* involvement in all cases diagnosed as CD. The heterogeneity and variability in clinical manifestations of constitutional bone disorders leaves open the possibility that *SOX-9* is involved in other skeletal dysplasias.

By analogy with *SRY*, it has been suggested that *SOX genes* might act as transcription factors in developmental control pathways. Some *SOX/Sox* proteins have been shown to exhibit sequence-specific binding (Harley, V.R. *et al.*, 1992, Science, **255**, 453-456; Denny, P. *et al.*, 1992, EMBO J, **11**, 3705-3712; van de Wetering, M. *et al.*, 1993, EMBO J, **12**, 3847-3854) and the C-terminal third of the *SOX-9* protein has a proline- and glutamine-rich region, similar to activation domains present in some transcription factors (Mitchell, P.J. *et al.*, 1989, Science, **245**, 371-378). This region would be missing in products translated from the mutated sequences present in the patients described in this report. The expression pattern of mouse *Sox-9* is consistent with a role in regulating mesenchymal cell differentiation to chondrocytes as discussed above.

Mutations in *SOX-9* causing male to female sex reversal in 46,XY individuals could be acting either before or after *SRY* in the sex determination pathway. The phenotype of 46,XY patients with mutations in *SRY* is usually female with complete gonadal dysgenesis. In a few cases, *SRY* mutations have been found to be inherited, with normal males and XY females occurring in the same family. These observations suggest that genes that perturb *SRY* function would result in either male or female, but probably no intersex development. Patients with CD show a spectrum of sexual phenotypes including partial masculinisation consistent with *SOX-9* having a role subsequent to *SRY* in the sex determination pathway.

SOX-9 is not the first mammalian gene to be shown to have a dosage sensitive role in sex determination. *DSS* causes male

to female sex reversal, with varying degrees of masculinisation, when present in two copies in 46,XY individuals. Absence of *DSS* is compatible with male development in the presence of *SRY* but it is not known if it is compatible with female development in 46,XX individuals. Because of the importance of *SOX-9* in bone formation, it is likely that nullisomy for *SOX-9* is lethal. *SOX-9* monosomy is compatible with ovarian development (Bridge, J. *et al.*, 1985, *supra*) and trisomy for 17q, including the region containing *SOX-9*, has not been associated with sex reversal (Lenzini, E. *et al.*, 1988, Ann. Genet, **31**, 175-180). The cause of the variability of sex reversal associated with CD remains to be determined. There is no obvious correlation between the severity of the skeletal anomalies and the incidence of sex reversal (Mansour, S., 1994, *supra*). The presence or absence of sex reversal in XY individuals may be determined by the nature of the mutation, or could lie in allelic differences at other loci.

The dosage sensitivity of *SOX-9* in sex determination and its sequence similarity to *SRY* suggest a possible evolutionary relationship between the two genes. It is plausible that a primordial dosage dependent sex determination system evolved into a dominant induction system by alteration of *SOX-9* or another *SOX* gene (Foster, J.W., *et al.*, 1994, *supra*). The mutated gene could function as a dominant inducer by becoming constitutively expressed and thus, when present, increasing dosage to be above a threshold required for male development.

There is a large body of indirect evidence suggesting that the sex determining function of *SRY* is expressed in pre-Sertoli cells in the developing gonadal ridge (Goodfellow, P.N. *et al.*, 1993, Ann. Rev. Genet., 27, 71-92). *SOX-9* could be required in these cells and *SRY* and *SOX-9* interactions may be required for full cell function. Another possibility is that *SOX-9* expression is required in a cell type that interacts with *SRY*-expressing pre-Sertoli cells to form testis. It is known that mesenchymal cells migrate from the mesonephros

underlying the genital ridge and that these migratory cells are required for testis formation (Wheater, P.R. *et al.*, 1979, Functional Histology (Churchill Livingstone, Edinburgh) and this might provide the link between CD and sex reversal. The identification of *SOX-9* as a gene mutated in both CD and autosomal sex reversal provides new tools for studying bone formation and sex determination.

TABLE

TABLE 1 Nucleotide homology of mouse, human and chicken *Sox-9*

COMPARISON	REGION A (nts 302-607)	REGION B (nts 844-1321)	REGION C (nts 1431-1822)	CODING REGION OVERALL
Mouse x Human	94.8%	90.0%	90.8%	91.3%
Mouse x Chicken	85.4%*	79.8%	79.7%	79.3%
Human x Chicken	86.2%*	84.5%	81.5%	82.4%

LEGENDS

TABLE 1

* Figures shown are for nts 484-607 due to unavailability of full chicken sequence.

Numbers in parentheses indicate nucleotide positions in mouse *Sox-9* sequence herein described.

FIG. 1

Nucleotide and predicted amino acid sequence of the mouse *Sox-9* cDNA. The 2249 base- pair sequence reveals an open reading frame that potentially encodes a protein of 507 amino acids from the first methionine codon. There are five methionine codons (indicated in italics) upstream of the HMG box (boxed), but only the fourth of these is associated with a strong consensus sequence for initiation of translation (Kozak, 1989, J. Cell Biol., **108**, 229). These five methionine codons are all conserved in the human *Sox-9* homologue (*SOX9*) sequence where they are also preceded by an in-frame stop codon (Foster *et al.*, in press) A glutamine- and proline-rich region extends from amino acid position 339 to 507. There are multiple stop codons (not marked) following the end of the coding sequence and a putative poly-adenylation signal is indicated in lower case lettering. The positions of introns are indicated by arrows; these were determined by comparison of cDNA and genomic DNA sequences.

Methods: λgt10 10 dpc (Clontech) and λSHlox 11.5 dpc (Invitrogen) mouse embryo cDNA libraries and a λFIX II mouse 129SV genomic library (Gubbay *et al.*, 1990, Nature, 346, 245-250), were screened for *Sox-9* clones using a *Sox-9* HMG box (Wright *et al.*, 1993, Nucleic Acids Research, 21, 744) and subsequently non-box probes under highly stringent conditions. Sequence of cDNA clones were obtained from both strands in nested deletions. Sequencing was performed using a USB Sequenase kit and results were confirmed using a PRISM Ready Reaction DyeDeoxy Terminator Cycle

Sequencing Kit and an Applied Biosystems DNA Sequencing System.

FIG. 2

Northern blot analysis of *Sox-9* expression in mouse embryos. Poly(A)$^+$ RNA isolated from whole embryos at 8.5, 9.5, 10.5, 11.5, 12.5 and 13.5 dpc was hybridised with a *Sox-9*-specific probe (upper panel) and a probe for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*: lower panel).

Methods: Poly (A)$^+$ RNA was prepared from whole embryos using a Pharmacia QuickPrep mRNA Purification kit. Northern analysis (Sambrook *et al.*, 1989, J. Molecular Cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor) was carried out using approximately 0.5 μg of each mRNA sample per lane. Following autoradiography, membranes were stripped of *Sox-9* probe and re-hybridised with a ^{32}P-labelled *Gapdh* probe to indicate the relative levels of mRNA in each lane. Transcript size was assessed by comparison to GIBCO-BRL 0.24-9.5 kb RNA ladder.

FIG. 3

Wholemount *in situ* hybridisations and alcian blue cartilage staining showing expression of *Sox-9* and cartilage matrix deposition in developing embryos:-

a. 9.5 dpc whole embryo showing *Sox-9* expression in the first branchial arch (b1), rostral somites (so), otocyst (oc) and some surface ectodermal cells overlying the spinal cord (se);

b. Partial view of a 10 dpc embryo showing expression within the caudal somites (so) and ventricular cells of the forebrain (vc);

c. 10.5 dpc whole embryo showing initiation of expression in the limb buds (lb) and in the second branchial arch (b2);

d. 10.5 dpc embryo stained with alcian blue dye. No cartilage is present at this stage, confirming that

cartilage formation is preceded by *Sox-9* expression;

e. 11.5 dpc showing advancement of expression in the limb buds, and onset in the scapula (s) and pelvis (p);

f. 12.5 dpc embryo showing staining in most skeletal structures;

g. alcian blue-stained 12.5 dpc embryo showing the cartilagenous skeleton at this stage; the otocyst, digits (d) and ribs (r) are indicated;

h. dorsal view of a 12.5 dpc embryo illustrating expression in ventricular cells of the spinal cord (vc); the otocysts are also indicated;

i. partial view of a 13.5 dpc embryo demonstrating that expression has progressed to the tips of the digits and the tail tip (t) where the cartilage is still being actively laid down but is switched off in more mature cartilage; staining is also seen in the vibrissae (v) at this stage.

Methods: Wholemount *in situ* hybridisations, using antisense and sense (not shown) RNA probes prepared from sub-clones of *Sox-9* gene sequence 3' to the HMG box but not containing any HMG box or poly-A-tail sequences, were carried out according to Wilkinson *et al.*, 1993, Methods Enzymol., **225**, 361-373. Cartilagenous tissue in whole 10.5 and 12.5 dpc embryos was stained according to a protocol modified from Ojeda *et al.*, 1970, Stain. Technol., **45**, 137-138. Stained specimens were photographed on an Olympus stereomicroscope using Kodak Ektachrome film.

FIG. 4

Wholemount *in situ* hybridisation of chrondrocytes in sections of mouse bone eight days post experimental fracture using anti-sense RNA probes (not shown) prepared from sub-clones of

mouse *Sox-9* gene sequences.

FIG. 5

Mapping of *Sox-9*. The approximate position of *Sox-9* with respect to the markers *D11Mit10* and *D11Mit36*, as indicated by a combination of interspecific backcross linkage data and haplotype analysis, is shown by bars A and B on the consensus linkage map of mouse chromosome 11 (Buchberg *et al.*, 1993, Mammal. Genome., **4**, S164-S175). A; *Sox-9* position relative to *D11Mit10* and B; relative to *D11Mit36*. The relative locations of *Sox-9* and *Tail-short (Ts)* cannot represented accurately as they were mapped relative to different markers in separate backcrosses. The locations of the neurological mutations *Jackson shaker (js), teetering (tn)* and *cerebellar outflow degeneration (Cod)* are also indicated. Genetic distance from the centromere is indicated in centiMorgans.

Methods: A gene-specific, single-copy cDNA probe was isolated from the region of *Sox-9* 3' to the HMG box and this probe was used to identify a restriction fragment length variant between the two mouse species *Mus spretus* and *Mus musculus domesticus* using the enzyme *PvuII* (data not shown). Mapping was carried out by analysing the segregation of these variants relative to known markers in a subset of interspecific backcross progeny mice (The European Backcross Collaborative Group, 1994, Human Mol. Genet., **3**, 621-627).

FIG. 6

Radiation hybrid map of 17q across the translocation breakpoint in patient E. STS markers are written vertically above a solid bar representing genomic DNA. The markers flanking the translocation breakpoint are indicated. Below, flanking STS markers *D17S970* and *SOX-9* tested on the B1 hybrid by PCR showing their absence/presence respectively. B1 is an L-M Tk^- somatic cell hybrid containing the translocation chromosome 2pter-q35:17q23-qter from patient E; PCTBA1.8 is a mouse somatic cell hybrid containing human

chromosome 17 only; HFL is a human fibroblast; L-M TK$^-$ is a mouse fibroblast.

Methods: The whole genome irradiation and fusion hybrids (WG-RH) were constructed by fusing A23 hamster fibroblasts with irradiated (6000 rads) HFL human fibroblasts (Walter, M.A. *et al.*, 1994, *supra*). The STS order was determined using the RHMAP programmes (Boehnke, M. *et al.*, 1991, Am. J. Hum. Genet, **49**, 1174-1188). PCR reactions were performed with 50 ng of genomic DNA, 1.5 mM $MgCl_2$ (2.5 mM $MgCl_2$ for *SOX-9* primers), 50 mM Kcl, 0.1% Triton-X100, 10 mM Tris-Cl (pH 8.5), 1.5 U *Taq* polymerase and 1 μM each primer. Thermocycling parameters were 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 60 seconds, then 5 mins at 72°C. The presence or absence of each STS in each WG-RH was determined by electrophoresis through ethidium bromide stained agarose gels. Primer sequences, AFMa346xg5-A, 5'CCAAAGTCCTAAAGGTGGG3'; AFMa346xg5-B, 5'TTTCAGGCAAATAAGGCAG3'; AFM189yb8-A, 5'TGGCAATCTAACAGATGAGA3'; AFM189yb8-B, 5'TCNCAAATGTCATATATCCA3'; SOX9-A, 5'AGTCCAGATTGACTGGAACACA; SOX9-B, 5'GCAATAAGATACTAATATGTAGAG3' D17S40-A, 5'GTCAGCAGAAATCCTAAAGG3'; D17S40-B, 5'GACTAATGCCGATGGTTAAG3'. The other primer sequences are available through the genome data base (GDB).

FIG. 7

Relationship between the chromosome 17 radiation hybrid map, YAC contig and cosmid contig for the region of the Patient E translocation breakpoint. Markers are indicated vertically above a solid bar representing genomic DNA. YACs are positioned below: solid bars indicate confirmed marker content, dashed lines represent the possible extent of the YAC. Sizes indicated are for the entire YAC and may include non-chromosome 17 sequences present

due to chimerism. The cosmid walk is shown below an expansion of the breakpoint region genomic DNA. The organisation and orientation of *SOX-9* are indicated. ICRF Reference Library YAC and cosmids (Lehrach, H. *et al.*, 1990, *supra*) are indicated as such, all other YACs are from Centre d'Etude du Polymorphisme Humain (Cohen, D. *et al.*, 1993, *supra*).

Methods: YAC and cosmid ends were isolated by vectorette PCR (Riley, J. *et al.*, 1990, Nucleic Acids Res., 18, 2887-2890) using the published YAC primers and cosmid vector (Lawrist4) primers LAW4L: C G C C T C G A G G T G G C T T A T C G and L A W 4 R: ATCATACACATACGATTTAGGTGAC.

FIG. 8a Nucleotide and predicted amino acid sequence of *SOX-9*. Numberings is with respect to the A in the first methionine codon of the open reading frame. An in-frame 5' stop codon and the predicted termination stop codon are in bold. The HMG box is boxed and the proline- and glutamine-rich region is underlined. The locations of the introns are indicated with arrows and a potential polyadenylation signal is indicated by bold, italic letters.

FIG. 8b Genomic organisation of the *SOX-9* gene. The solid bar represents genomic DNA. The *SOX-9* exons are boxed and the HMG box cross hatched. The positions of the introns are indicated.

Methods: Initial cDNA clones were obtained by screening a lambda gt 10 human testis library (Clontech) using a *SOX-A* box probe (Stevanovic, M. *et al.*, 1993, *supra*). A composite transcript was determined from these overlapping clones and from further clones obtained from an HT1080 (fibrosarcome) cDNA library (a kind gift of D. L. Simmons) and a human foetal brain library (HGMP Resource Centre, Harrow). Sequencing was performed using the dideoxy chain termination method. The location of the intron/exon boundaries was determined by restriction mapping of genomic and cDNA clones and by comparison of the genomic and CDNA sequences. Initial localisation of the *SOX-9* cDNA to chromosome 17 was determined

by probing a somatic cell hybrid panel. Sublocalisation to 17q23-qter was determined using a panel of chromosome 17 deletion hybrids including PCTBA1.8, TRID62, PLT8, PJT2A1 and DCR1 (Black, D.M. *et al.*, 1993, E. Am. J. Hum. Genet., 52, 702-710) and refined to 17q24 by fluorescence *in situ* hybridisation to normal human metaphase spreads.

FIG. 9

Diagrammatic representation of mouse *Sox-9* gene structure. Numerals above the line denote the nucleotide position of the mouse *Sox-9* gene having regard to the DNA sequence shown in FIG. 1. The gene comprises a 5' untranslated region (nts 1-301), region A (nts 302-607), a HMG box (nt 608-843), region B (nts 844-1321), P/Q/A - rich region (nts 1322-1429), region C (nts 1430-1822) and the 3'untranslated region (nts 1823-2249).

FIG. 10

Single-strand conformation polymorphism (SSCP) and sequence analysis of *SOX-9* in campomelic dysplasia patients.

FIG. 10a *SOX-9* open reading frame (shaded boxes) showing the HMG box (heavy shading). Numbers indicate nucleotide sequence beginning with the A of the first methionine, with introns occurring after nucleotides 431 and 685. Solid bars below indicate regions of the ORF generating unique SSCP conformers. Positions of mutations are indicated by arrows.

FIG. 10b SSCP using primers indicated in (a). Lane 1; patient DNA. For Patients S.H. and G., lanes 2 and 3 are DNAs from father and mother, respectively. For Patient A.H., lanes 2 and 3 are DNAs from unrelated (normal) individuals.

FIG. 10c Sequencing gels of normal and mutated patient alleles. The position of each mutation is indicated. Sequence for Patients S.H. and A.H. is the coding strand; Patient G. sequence is the non-coding strand.

Methods: Primer sequences: 534,

5'GAGGAAGTCGGTGAAGAAC3'; 661, 5'TCGCTCATGCCGGAGGAGGAG3'; 687, 5'GCAATCCCAGGGCCCACCGAC3'; 854, 5'TTGGAGATGACGTCGACTGCTC3'; 836, 5'GCAGCGACGTCATCTCCAAC3'; 1018,5'GCTGCTTGGACATCCACACGT3'. PCRs (10 μl) were performed as in FIG. 1 with the non-radioactive dCTP concentration reduced to 1/10 and the addition of 0.05 μl of [α-^{33}P]dCTP (1000-3000 Ci mmol^{-1}, 10 mCi ml^{-1}) and 0.2 μM of each primer. Reactions were cycled for 30 sec at 94°C, 30 sec at 65°C (534-661 and 836-1018) or 70°C (687-854), 45 sec at 72°C for 35 cycles. PCR products were denatured by adding 10 μl of 0.2% SDS, 20 mM EDTA then 10 μl 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and heating to 100°C for 5 min. Two μl were loaded onto 6% acrylamide:Bis-acrylamide (37.5:1), 5% glycerol gels. Electrophoresis was carried out at 25 W at 4°C. PCR products from duplicate reactions were subcloned and at least 10 clones from each were sequenced by either the dideoxy chain termination method or by DyeDeoxy Terminator Cycle Sequencing (ABI). DNA profiling of each family using 12 chromosome 8 microsatellite markers (heterozygosity > 70%) showed no discordant results between parents and offspring.

CLAIMS

1. An isolated DNA molecule comprising a DNA sequence selected from a group consisting of:

(i) a sequence of nucleotides as shown in FIG. 1;

(ii) a sequence complementary to the sequence according to (i); and

(iii) a sequence having up to 21% variation from the sequences according to (i) or (ii) which sequence is capable of hybridising thereto under standard hybridisation conditions which codes for a polypeptide of the SOX-9 type.

2. An isolated DNA molecule comprising a DNA sequence selected from a group consisting of:

(a) a sequence of nucleotides as shown in FIG. 8a;

(b) a sequence complementary to the sequence according to (a); and

(c) a sequence having up to 18% variation from the sequences according to (a) or (b) which sequence is capable of hybridising thereto under standard hybridisation conditions and which codes for a polypeptide of the SOX-9 type.

3. A recombinant protein when encoded by a DNA sequence as defined in Claim 1.

4. A recombinant protein when encoded by a DNA sequence as defined in Claim 2.

5. A recombinant protein comprising an amino acid sequence as shown in FIG. 1 as well as polypeptides of the SOX-9 type containing 93.5% - 100% identity to said sequence.

6. A recombinant protein comprising an amino acid sequence as shown in FIG. 8a as well as polypeptides containing 93.5% - 100% identity to said sequence.

7. A method of regeneration of bone or cartilage by administration of a DNA molecule as claimed in Claim 1.

8. A method of regeneration of bone or cartilage by

administration of a DNA molecule as claimed in Claim 2.

9. A method of regeneration of bone or cartilage by administration of a recombinant protein as claimed in Claim 3.

10. A method of regeneration of bone or cartilage by administration of a recombinant protein as claimed in Claim 4.

Human SOX9 cDNA sequence

```
   1  CGGAGCTCGA AACTGACTGG AAACTTCAGT GGCGCGGAGA CTCGCCAGTT
  51  TCAACCCCGG AAACTTTTCT TTGCAGGAGG AGAAGAGAAG GGGTGCAAGC
 101  ACCCCCACTT TTACTCTTTT TCCTCCCCTC CTCCTCCTCT CCAATTCGCC
 151  TCCCCCCACT TGGAGCGGGC AGCTGTGAAC TGGCCACCCC GCGCCTTCCT
 201  AAGTGCTCGC CGCGGTAGCC GGCCGACGCG CCAGCTTCCC CGGGAGCCGC
 251  TTGCTCCGCA TCCGGGCAGC CGAGGGGAGA GGAGCCCGCG CCTCGAGTCC
 301  CCGAGCCGCC GCGGCTTCTC GCCTTTCCCG GCCACCAGCC CCCTGCCCCG
 351  GGCCCGCGTA TGAATCTCCT GGACCCCTTC ATGAAGATGA CCGACGAGCA
 401  GGAGAAGGGC CTGTCCGGCG CCCCCAGCCC CACCATGTCC GAGGACTCCG
 451  CGGGCTCGCC CTGCCCGTCG GGCTCCGGCT CGGACACCGA GAACACGCGG
 501  CCCCAGGAGA ACACGTTCCC CAAGGGCGAG CCCGATCTGA AGAAGGAGAG
 551  CGAGGAGGAC AAGTTCCCCG TGTGCATCCG CGAGGCGGTC AGCCAGGTGC
 601  TCAAAGGCTA CGACTGGACG CTGGTGCCCA TGCCGGTGCG CGTCAACGGC
 651  TCCAGCAAGA ACAAGCCGCA CGTCAAGCGG CCCATGAACG CCTTCATGGT
 701  GTGGGCGCAG GCGGCGCGCA GGAAGCTCGC GGACCAGTAC CCGCACTTGC
 751  ACAACGCCGA GCTCAGCAAG ACGCTGGGCA AGCTCTGGAG ACTTCTGAAC
 801  GAGAGCGAGA AGCGGCCCTT CGTGGAGGAG GCGGAGCGGC TGCGCGTGCA
 851  GCACAAGAAG GACCACCCGG ATTACAAGTA CCAGCCGCGG CGGAGGAAGT
 901  CGGTGAAGAA CGGGCAGGCG GAGGCAGAGG AGGCCACGGA GCAGACGCAC
 951  ATCTCCCCCA ACGCCATCTT CAAGGCGCTG CAGGCCGACT CGCCACACTC
1001  CTCCTCCGGC ATGAGCGAGG TGCACTCCCC CGGCGAGCAC TCGGGGCAAT
1051  CCCAGGGCCC ACCGACCCCA CCCACCACCC CCAAAACCGA CGTGCAGCCG
1101  GGCAAGGCTG ACCTGAAGCG AGAGGGGCGC CCCTTGCCAG AGGGGGGCAG
1151  ACAGCCCCCT ATCGACTTCC GCGACGTGGA CATCGGCGAG CTGAGCAGCG
1201  ACGTCATCTC CAACATCGAG ACCTTCGATG TCAACGAGTT TGACCAGTAC
1251  CTGCCGCCCA ACGGCCACCC GGGGGTGCCG GCCACGCACG GCCAGGTCAC
1301  CTACACGGGC AGCTACGGCA TCAGCAGCAC CGCGGCCACC CCGGCGAGCG
1351  CGGGCCACGT GTGGATGTCC AAGCAGCAGG CGCCGCCGCC ACCCCCGCAG
1401  CAGCCCCCAC AGGCCCCGCC GGCCCCGCAG GCGCCCCCGC AGCCGCAGGC
1451  GGCGCCCCCA CAGCAGCCGG CGGCACCCCC GCAGCAGCCA CAGGCGCACA
```

Continued over...

FIG. 1

Human SOX9 cDNA sequence (continued)

1501 CGCTGACCAC GCTGAGCAGC GAGCCGGGCC AGTCCCAGCG AACGCACATC

1551 AAGACGGAGC AGCTGAGCCC CAGCCACTAC AGCGAGCAGC AGCAGCACTC

1601 GCCCCAACAG ATCGCCTACA GCCCCTTCAA CCTCCCACAC TACAGCCCCT

1651 CCTACCCGCC CATCACCCGC TCACAGTACG ACTACACCGA CCACCAGAAC

1701 TCCAGCTCCT ACTACAGCCA CGCGGCAGGC CAGGGCACCG GCCTCTACTC

1751 CACCTTCACC TACATGAACC CCGCTCAGCG CCCCATGTAC ACCCCCATCG

1801 CCGACACCTC TGGGGTCCCT TCCATCCCGC AGACCCACAG CCCCCAGCAC

1851 TGGGAACAAC CCGTCTACAC ACAGCTCACT CGACCTTGAG GAGGCCTCCC

1901 ACGAAGGGCG ACGATGGCCG AGATGATCCT AAAAATAACC GAAGAAAGAG

1951 AGGACCAGAA TTCCCTTTGG ACATTTGTGT TTTTTTGTTT TTTTATTTTG

2001 TTTTGTTTTT TCTTCTTCTT CTTCTTCCTT AAAGACATTT AAGCTAAAGG

2051 CAACTCGTAC CCAAATTTCC AAGACACAAA CATGACCTAT CCAAGCGCAT

2101 TACCCACTTG TGGCCAATCA GTGGCCAGGC CAACCTTGGC TAAATGGAGC

2151 AGCGAAATCA ACGAGAAACT GGACTTTTTA AACCCTCTTC AGAGCAAGCG

2201 TGGAGGATGA TGGAGAATCG TGTGATCAGT GTGCTAAATC TCTCTGCCTG

2251 TTTGGACTTT GTAATTATTT TTTTAGCAGT AATTAAAGAA AAAAGTCCTC

2301 TGTGAGGAAT ATTCTCTATT TTAAATATTT TTAGTATGTA CTGTGTATGA

2351 TTCATTACCA TTTTGAGGGG ATTTATACAT ATTTTTAGAT AAAATTAAAT

2401 GCTCTTATTT TTCCAACAGC TAAACTACTC TTAGTTGAAC AGTGTGCCCT

2451 AGCTTTTCTT GCAACCAGAG TATTTTTGTA CAGATTTGCT TTCTCTTACA

2501 AAAAAAAAAA AAAA end

FIG. 1

Human SOX9 protein sequence

```
  1  MNLLDPFMKM TDEQEKGLSG APSPTMSEDS AGSPCPSGSG SDTENTRPQE
 51  NTFPKGEPDL KKESEEDKFP VCIREAVSQV LKGYDWTLVP MPVRVNGSSK
101  NKPHVKRPMN AFMVWAQAAR RKLADQYPHL HNAELSKTLG KLWRLLNESE
151  KRPFVEEAER LRVQHKKDHP DYKYQPRRRK SVKNGQAEAE EATEQTHISP
201  NAIFKALQAD SPHSSSGMSE VHSPGEHSGQ SQGPPTPPTT PKTDVQPGKA
251  DLKREGRPLP EGGRQPPIDF RDVDIGELSS DVISNIETFD VNEFDQYLPP
301  NGHPGVPATH GQVTYTGSYG ISSTAATPAS AGHVWMSKQQ APPPPPQQPP
351  QAPPAPQAPP QPQAAPPQQP AAPPQQPQAH TLTTLSSEPG QSQRTHIKTE
401  QLSPSHYSEQ QQHSPQQIAY SPFNLPHYSP SYPPITRSQY DYTDHQNSSS
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501  PVYTQLTRP*
```

FIG. 1

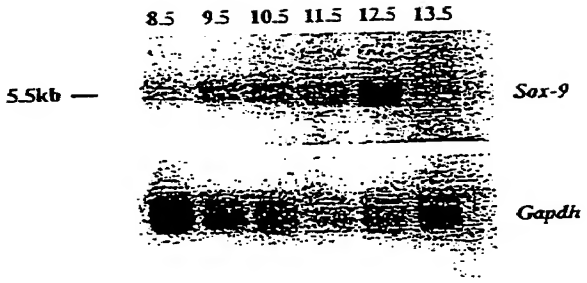


FIG. 2

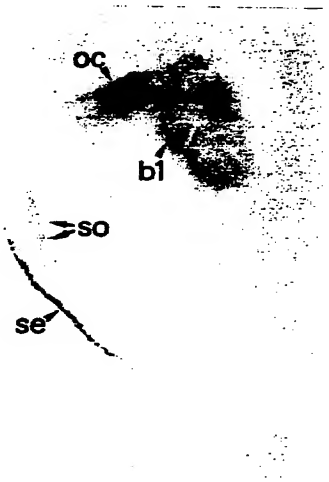


FIG. 3a

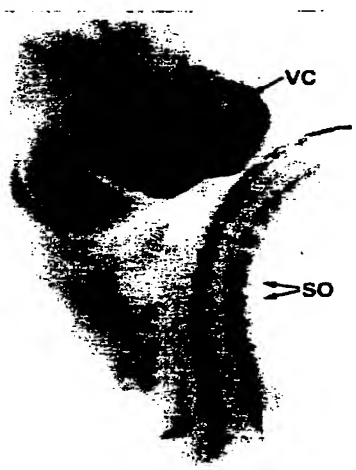


FIG. 3b

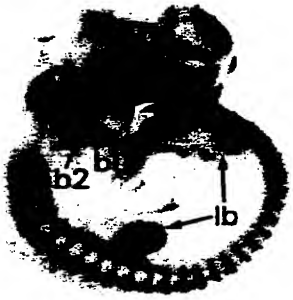


FIG. 3c

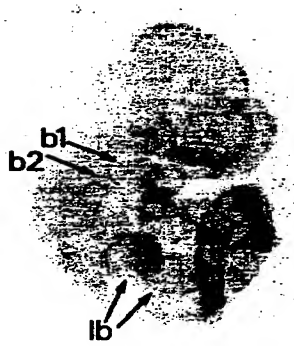


FIG. 3d

FIG. 3e

FIG. 3f

FIG. 3g

FIG. 3h

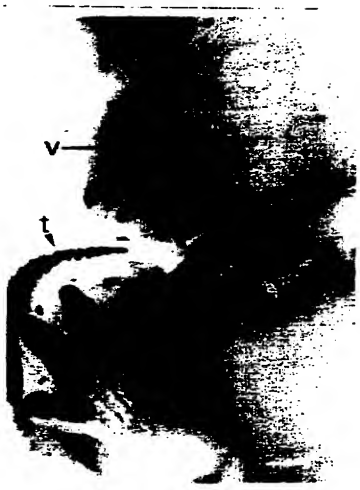


FIG 3i

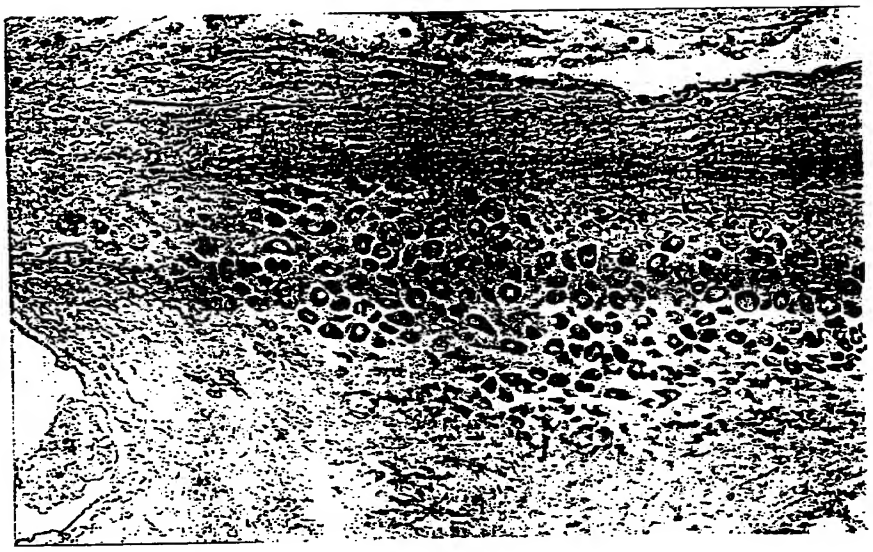


FIG. 4

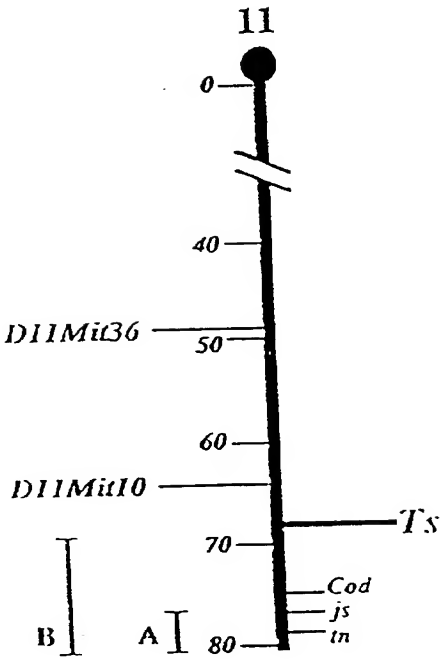
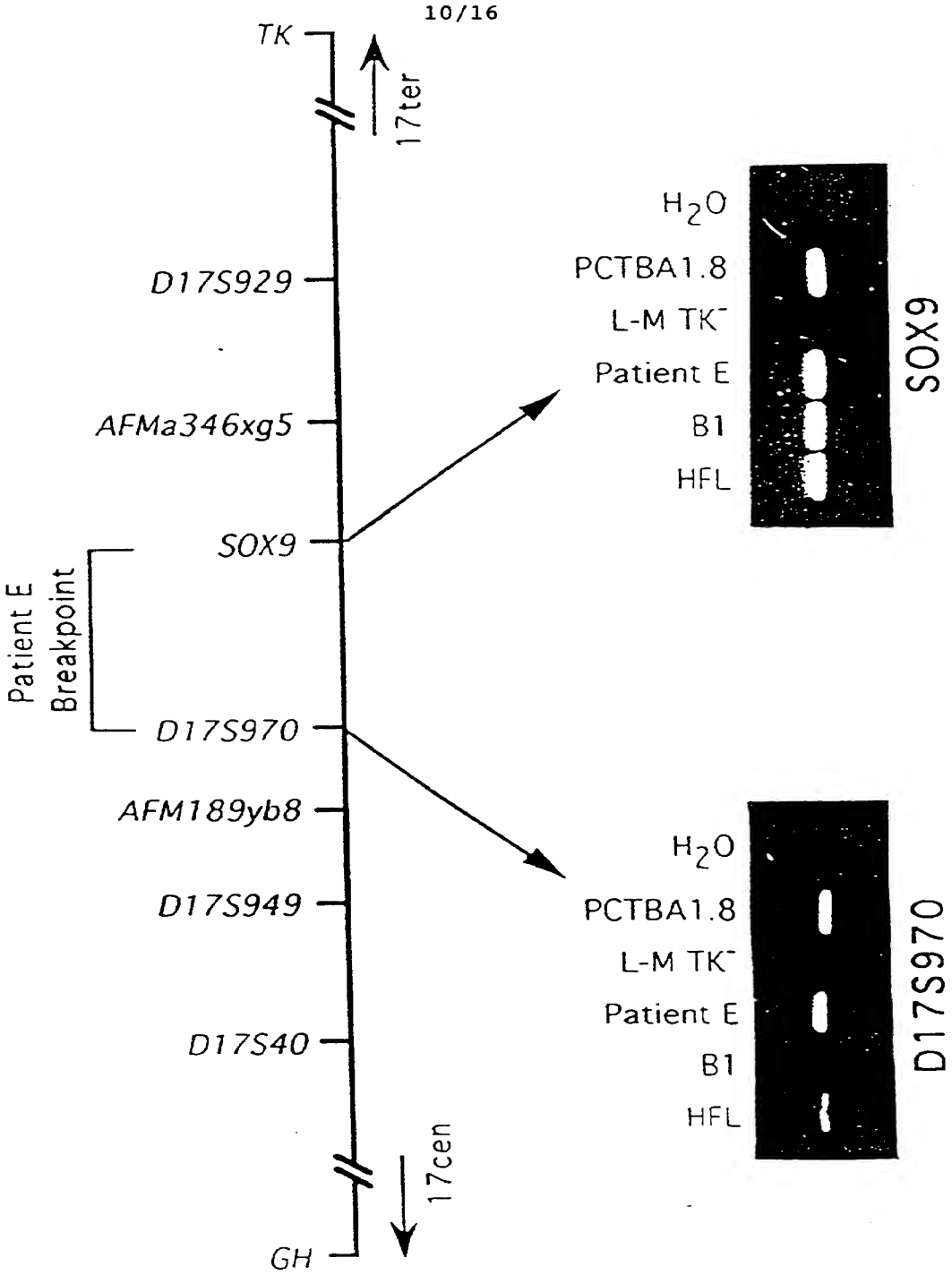


FIG. 5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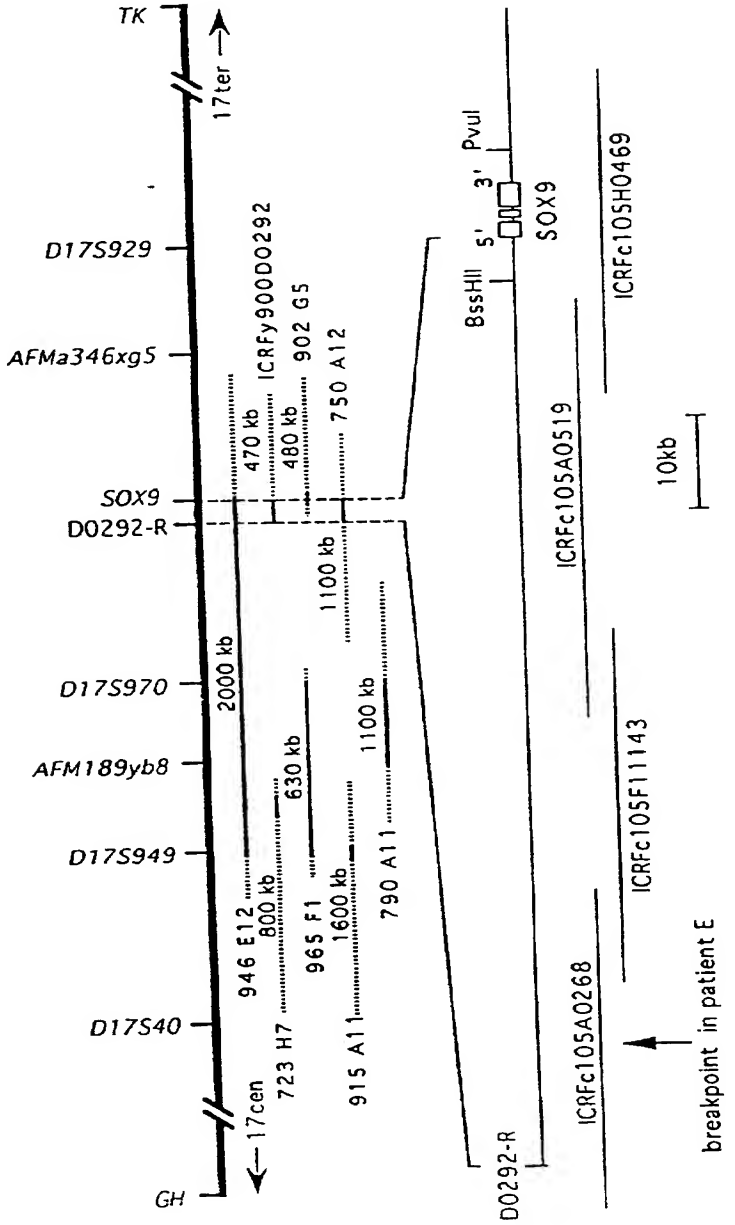
AFMa346xg5
SOX9
Patient E Breakpoint
D17S970
AFM189yb8
D17S949
D17S40
17cen
GH
H2O
PCTBA1.8
L-M TK-
Patient E
B1
HFL
SOX9
H2O
PCTBA1.8
L-M TK-
Patient E
B1
HFL
D17S970

FIG. 6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D17S929
AFMa346xg5
SOX9
D0292-R
D17S970
AFM189yb8
D17S949
D17S40
17cen
GH
2000 kb
946 E12
800 kb
723 H7
630 kb
965 F1
1600 kb
915 A11
1100 kb
790 A11
1100 kb
470 kb
ICRFy900D0292
480 kb
902 G5
750 A12
BssHII
5'
3'
PvuI
SOX9
ICRFc105H0469
ICRFc105A0519
10kb
ICRFc105F11143
ICRFc105A0268
breakpoint in patient E
D0292-R

FIG. 7

```
 CGGAGCTCGAAACTGACTGGAAACTTCAGTGGCGCGGAGACTCGCCAGTTTCAACCCCGGAAACTTTTCTTTGC  -285
AGGAGGAGAAGAGAAGGGGTGCAAGCGCCCCCACTTTTGCTCTTTTTCCTCCCCTCCTCCTCCTCTCCAATTCGC
CTCCCCCCACTTGGAGCGGGCAGCTGTGAACTGGCCACCCCGCGCCTTCCTAAGTGCTCGCCGCGGTAGCCGGCC
GACGCGCCAGCTTCCCCGGGAGCCGCTTGCTCCGCATCCGGGCAGCCGAGGGGAGAGGAGCCCGCGCCTCGAGTC
CCCGAGCCGCCGCGGCTTCTCGCCTTTCCCGGCCACCAGCCCCCTGCCCCGGGCCCGCGTATGAATCTCCTGGAC    15
                                                          M  N  L  L  D
CCCTTCATGAAGATGACCGACGAGCAGGAGAAGGGCCTGTCCGGCGCCCCCAGCCCCACCATGTCCGAGGACTCC    90
 P  F  M  K  M  T  D  E  Q  E  K  G  L  S  G  A  P  S  P  T  M  S  E  D  S
GCGGGCTCGCCCTGCCCGTCGGGCTCCGGCTCGGACACCGAGAACACGCGGCCCCAGGAGAACACGTTCCCCAAG   165
 A  G  S  P  C  P  S  G  S  G  S  D  T  E  N  T  R  P  Q  E  N  T  F  P  K
GGCGAGCCCGATCTGAAGAAGGAGAGCGAGGAGGACAAGTTCCCCGTGTGCATCCGCGAGGCGGTCAGCCAGGTG   330
 G  E  P  D  L  K  K  E  S  E  E  D  K  F  P  V  C  I  R  E  A  V  S  Q  V
CTCAAAGGCTACGACTGGACGCTGGTGCCCATGCCGGTGCGCGTCAACGGCTCCAGCAAGAACAAGCCGCACGTC   405
 L  K  G  Y  D  W  T  L  V  P  M  P  V  R  V  N  G  S  S  K  N  K  P  H  V
AAGCGGCCCATGAACGCCTTCATGGTGTGGGCGCAGGCGGCGCGCAGGAAGCTCGCGGACCAGTACCCGCACTTG   480
 K  R  P  M  N  A  F  M  V  W  A  Q  A  A  R  R  K  L  A  D  Q  Y  P  H  L
CACAACGCCGAGCTCAGCAAGACGCTGGGCAAGCTCTGGAGACTTCTGAACGAGAGCGAGAAGCGGCCCTTCGTG   555
 H  N  A  E  L  S  K  T  L  G  K  L  W  R  L  L  N  E  S  E  K  R  P  F  V
GAGGAGGCGGAGCGGCTGCGCGTGCAGCACAAGAAGGACCACCCGGATTACAAGTACCAGCCGCGGCGGAGGAAG   630
 E  E  A  E  R  L  R  V  Q  H  K  K  D  H  P  D  Y  K  Y  Q  P  R  R  R  K
TCGGTGAAGAACGGGCAGGCGGAGGCAGAGGAGGCCACGGAGCAGACGCACATCTCCCCCAACGCCATCTTCAAG   705
 S  V  K  N  G  Q  A  E  A  E  E  A  T  E  Q  T  H  I  S  P  N  A  I  F  K
GCGCTGCAGGCCGACTCGCCACACTCCTCCTCCGGCATGAGCGAGGTGCACTCCCCCGGCGAGCACTCGGGGCAA   780
 A  L  Q  A  D  S  P  H  S  S  S  G  M  S  E  V  H  S  P  G  E  H  S  G  Q
TCCCAGGGCCCACCGACCCCACCCACCACCCCCAAAACCGACGTGCAGCCGGGCAAGGCTGACCTGAAGCGAGAG   855
 S  Q  G  P  P  T  P  P  T  T  P  K  T  D  V  Q  P  G  K  A  D  L  K  R  E
GGGCGCCCCTTGCCAGAGGGGGGCAGACAGCCCCCTATCGACTTCCGCGACGTGGACATCGGCGAGCTGAGCAGC   930
 G  R  P  L  P  E  G  G  R  Q  P  P  I  D  F  R  D  V  D  I  G  E  L  S  S
GACGTCATCTCCAACATCGAGACCTTCGATGTCAACGAGTTTGACCAGTACCTGCCGCCCAACGGCCACCCGGGG  1005
 D  V  I  S  N  I  E  T  F  D  V  N  E  F  D  Q  Y  L  P  P  N  G  H  P  G
GTGCCGGCCACGCACGGCCAGGTCACCTACACGGGCAGCTACGGCATCAGCAGCACCGCGGCCACCCCGGCGAGC  1080
 V  P  A  T  H  G  Q  V  T  Y  T  G  S  Y  G  I  S  S  T  A  A  T  P  A  S
GCGGGCCACGTGTGGATGTCCAAGCAGCAGGCGCCGCCGCCACCCCCGCAGCAGCCCCCACAGGCCCCGCCGGCC  1155
 A  G  H  V  W  M  S  K  Q  Q  A  P  P  P  P  P  Q  Q  P  P  Q  A  P  P  A
CCGCAGGCGCCCCCCGCAGCCGCAGGCGGCGCCCCCACAGCAGCCGGCGGCACCCCCGCAGCAGCCACAGGCGCAC  1230
 P  Q  A  P  P  Q  P  Q  A  A  P  P  Q  Q  P  A  A  P  P  Q  Q  P  Q  A  H
ACGCTGACCACGCTGAGCAGCGAGCCGGGCCAGTCCCAGCGAACGCACATCAAGACGGAGCAGCTGAGCCCCAGC  1305
 T  L  T  T  L  S  S  E  P  G  Q  S  Q  R  T  H  I  K  T  E  Q  L  S  P  S
CACTACAGCGAGCAGCAGCAGCACTCGCCCCAACAGATCGCCTACAGCCCCTTCAACCTCCCACACTACAGCCCC  1380
 H  Y  S  E  Q  Q  Q  H  S  P  Q  Q  I  A  Y  S  P  F  N  L  P  H  Y  S  P
TCCTACCCGCCCATCACCCGCTCACAGTACGACTACACCGACCACCAGAACTCCAGCTCCTACTACAGCCACGCG  1455
 S  Y  P  P  I  T  R  S  Q  Y  D  Y  T  D  H  Q  N  S  S  S  Y  Y  S  H  A
GCAGGCCAGGGCACCGGCCTCTACTCCACCTTCACCTACATGAACCCCGCTCAGCGCCCCATGTACACCCCCATC  1530
 A  G  Q  G  T  G  L  Y  S  T  F  T  Y  M  N  P  A  Q  R  P  M  Y  T  P  I
GCCGACACCTCTGGGGTCCCTTCCATCCCGCAGACCCACAGCCCCCAGCACTGGGAACAACCCGTCTACACACAG  1605
 A  D  T  S  G  V  P  S  I  P  Q  T  H  S  P  Q  H  W  E  Q  P  V  Y  T  Q
CTCACTCGACCTTGAGGAGGCCTCCCACGAAGGGCGACGATGGCCGAGATGATCCTAAAAATAACCGAAGAAAGA  1680
 L  T  R  P
```

continue over ..

FIG. 8a

continued ...

GAGGACCAACCAGAATTCCCTTTGGACATTTGTGTTTTTTTGTTTTTTTATTTTGTTTTGTTTTTTCTTCTTCTT 1755
CTTCTTCCTTAAAGACATTTAAGCTAAAGGCAACTCGTACCCAAATTTCCAAGACACAAACATGACCTATCCAAG
CGCATTACCCACTTGTGGCCAATCAGTGGCCAGGCCAACCTTGGCTAAATGGAGCAGCGAAATCAACGAGAAACT
GGACTTTTTAAACCCTCTTCAGAGCAAGCGTGGAGGATGATGGAGAATCGTGTGATCAGTGTGCTAAATCTCTCT
GCCTGTTTGGACTTTGTAATTATTTTTTTAGCAGTAATTAAAGAAAAAAGTCCTCTGTGAGGAATATTCTCTATT
TTAAATATTTTTAGTATGTACTGTGTATGATTCATTACCATTTTGAGGGGATTTATACATATTTTTAGATAAAAT
TAAATGCTCTTATTTTTCCAACAGCTAAACTACTCTTAGTTGAACAGTGTGCCCTAGCTTTTCTTGCAACCAGAG
TATTTTTGTACAGATTTGCTTTCTCTTACAAAAAGAAAAAAAAAAATCCTGTTGTATTAACATTTAAAAACAGAAT
TGTGTTATGTGATCAGTTTTGGGGGTTAACTTTGCTTAATTCCTCAGGCTTTGCGATTTAAGGAGGAGCTGCCTT
AAAAAAAAATAAAGGCCTTATTTTGCAATTATGGGAGTAAACAATAGTCTAGAGAAGCATTTGGTAAGCTTTATG
ATATATATATTTTTTAAAGAAGAGAAAAACACCTTGAGCCTTAAAACGGTGCTGCTGGGAAACATTTGCACTCTT
TTAGTGCATTTCCTCCTGCCTTTGCTTGTTCACTGCAGTCTTAAGAAAGAGGTAAAAGGCAAGCAAAGGAGATGA
AATCTGTTCTGGGAATGTTTCAGCAGCCAATAAGTGCCCGAGCACACTGCCCCCGGTTGCCTGCCTGGGCCCCAT
GTGGAAGGCAGATGCCTGCTCGCTCTGTCACCTGTGCCTCTCAGAACACCAGCAGTTAACCTTCAAGACATTCCA
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CCAATTTTACCTTCTTTAAAATAGGTTGTTGGAGCTTTCCTCAAAGGGTATGGTCATCTGTTGTTAAATTATGTT
CTTAACTGTAACCAGTTTTTTTTTATTTATCTCTTTAATCTTTTTTATTATTAAAAGCAAGTTTCTTTGTATTCC
TCACCCTAGATTTGTATAAATGCCTTTTTGTCCATCCCTTTTTTCTTTGTTGTTTTTGTTGAAAACAAACTGGAA
ACTTGTTTCTTTTTTTGTATAAATGAGAGATTGCAAATGTAGTGTATCACTGAGTCATTTGCAGTGTTTTCTGCC
ACAGACCTTTGGGCTGCCTTATATTGTGTGTGTGTGTGGGTGTGTGTGTTTTGACACAAAAACAATGCAAGCA
TGTGTCATCCATATTTCTCTACATCTTCTCTTGGAGTGAGGGAGGCTACCTGGAGGGGATCAGCCCACTGACAGA
CCTTAATCTTAATTACTGCTGTGGCTAGAGAGTTTGAGGATTGCTTTTTAAAAAAGACAGCAAACTTTTTTTTTT
ATTTAAAAAAAAGATATATTAACAGTTTTAGAAGTCAGTAGAATAAAATCTTAAAGCACTCATAATATGGCATCCT
TCAATTTCTGTATAAAAGCAGATCTTTTTAAAAAAGATACTTCTGTAACTTAAGAAACCTGGCATTTAAATCATA
TTTTGTCTTTAGGTAAAAGCTTTGGTTTGTGTTCGTGTTTTGTTTGTTTCACTTGTTTCCCTCCCAGCCCCAAAC
CTTTTGTTCTCTCCGTGAAACTTACCTTTCCCTTTTTCTTTCTCTTTTTTTTTTTTGTATATTATTGTTTAC**AATA**
AATATACATTGCATTAAAAAGAAAAAAAAAAAAA 3634

FIG. 8a

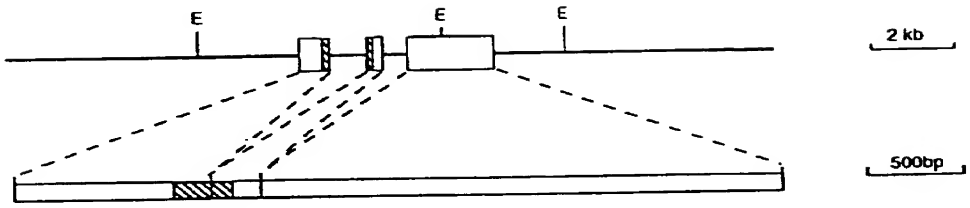

FIG 8b

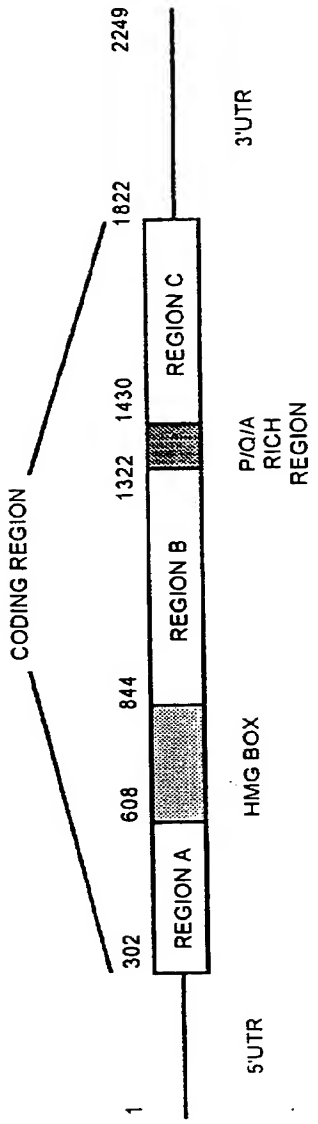
2249
1822
1430
1322
844
608
302
1
REGION C
REGION B
REGION A
3'UTR
P/Q/A RICH REGION
HMG BOX
5'UTR
CODING REGION

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00799

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/12, C07K 14/47, A61K 48/00, 38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : C12N 15/12 C07K 14/47

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT: sox( )9 or sox9 or sox
CHEMICAL ABSTRACTS, GENBANK: as above and sequence search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NATURE GENETICS, Vol. 9, no. 1, (1995), WRIGHT, E. et al. "The Sry-related gene Sox-9 is expressed during chondrogenesis in mouse embryos", pages 15-20. Whole document	1-10
P,X	NATURE, Vol. 372, (8 December 1994), Foster, J.W. et al. "Campomelic dysplasia and autosoma sex reversal caused by mutations in an SRY-related gene", pages 525-530.	1-10

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
16 February 1996

Date of mailing of the international search report
7th March 1996

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
PO BOX 200
WODEN ACT 2606
AUSTRALIA Facsimile No.: (06) 285 3929

Authorized officer

JENNIFER POTTER

Telephone No.: (06) 283 2247

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INTERNATIONAL SEARCH REPORT

Ir.. .national Application No.
PCT/AU 95/00799

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, Vol. 21, No. 12 (1993), GOZÉ, C. et al. "Partial Cloning of SOX-11 and SOX-12, two new human SOX genes", page 2943. Figure 1	1-10
X	NUCLEIC ACIDS RESEARCH, vol. 21, No. 3 (1993), WRIGHT, E.M., et al. "Seven new members of the Sox gene family expressed during mouse development", page 744. Figure 1	1-10
X	NUCLEIC ACIDS RESEARCH, Vol. 20, No. 11 (1992), DENNY, P. Et al. "A conserved family of genes related to the testis determining gene, SRY", page 2887. Figure 1	1-10
A	THE JOURNAL OF ENDOCRINOLOGY, Vol. 147, No. 2 (1995), HAWKINS, J.R. "Genetics of XY sex reversal", pages 183-187. Pages 186-187	1-10